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Ankyrin Repeat Proteins Comprise a Diverse Family of Bacterial Type IV Effectors[#]

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Abstract

Specialized secretion systems are used by many bacteria to deliver effector proteins into host cells that can either mimic or disrupt the function of eukaryotic factors. We found that the intracellular pathogens *Legionella pneumophila* and *Coxiella burnetii* use a type IV secretion system to deliver into eukaryotic cells a large number of different bacterial proteins containing ankyrin repeat homology domains called Anks. The *L. pneumophila* AnkX protein prevented microtubule-dependent vesicular transport to interfere with fusion of the *L. pneumophila*-containing vacuole with late endosomes after infection of macrophages, demonstrating that Ank proteins possess effector functions important for bacterial infection of eukaryotic host cells.

Type IV secretion systems (TFSSs) are molecular machines used by gram-negative bacteria for protein transfer into recipient cells (1). Many bacterial pathogens and endosymbionts use TFSSs to regulate host processes important for survival and replication (2), and several of these organisms have a large number of genes encoding proteins with multiple Ankyrin Repeat Homology Domains (ARHDs) (3–7). Infrequently encountered in bacterial proteins but common in eukaryotic proteins, ARHDs form molecular scaffolds that mediate protein-protein interactions (8). An *Anaplasma phagocytogeticum* protein containing multiple ARHDs called Anka (9), and several ARHD proteins in strains of *Wolbachia* (10,11), have been proposed to be delivered into host cells by a TFSS (12); however, whether Ank proteins are *bona fide* TFSS effectors has not been established.

Legionella pneumophila and *Coxiella burnetii* are both intracellular pathogens that encode several proteins containing ARHDs and a TFSS called Dot/Icm (5–7). To test whether ARHD proteins are TFSS substrates, we measured host cell translocation of four *L. pneumophila* Ank proteins fused to a calmodulin-dependent adenylate cyclase reporter (Cya), using the *L. pneumophila* effector RalF as a positive control (13,14). These four Ank proteins were delivered into mammalian cells as indicated by a greater than ten-fold increase in cAMP following infection (Fig. 1A). No cAMP increase was observed when the Cya-Ank proteins were produced in the *L. pneumophila* *ΔdotA* mutant lacking a functional TFSS, indicating that the Dot/Icm system is required for Ank protein delivery into host cells. Thirteen different C.

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burnetii proteins with ARHDs were tested for translocation using the Cya assay. Genetic manipulation of the obligate intracellular pathogen *C. burnetii* is not currently possible, but the *C. burnetii* and *L. pneumophila* Dot/Icm systems are functionally similar (15,16), suggesting that *L. pneumophila* will deliver *C. burnetii* Dot/Icm substrates into host cells. *C. burnetii* AnkA, AnkB, AnkF and AnkG fusion proteins were efficiently translocated into host cells by a process requiring the *L. pneumophila* Dot/Icm system (Fig. 1B). The AnkE, AnkH, AnkI and AnkM proteins were delivered less efficiently (Fig. 1B). Specific Ank-encoding RNA transcripts were expressed during *C. burnetii* infection (Fig. 1C), suggesting protein products should be available for delivery into host cells by the *C. burnetii* Dot/Icm system.

An antibody generated against AnkF confirmed that this protein was secreted during *C. burnetii* infection of mammalian cells (Fig. 1D), whereas, the *C. burnetii* translation factor EF-Ts was not and remained associated with bacteria in the pellet. The amount of secreted AnkF diminished over time upon treatment with the bacterial protein synthesis inhibitor chloramphenicol, which did not measurably affect levels of AnkF associated with bacterial cells (Fig. 1D). The short half-life of secreted AnkF revealed by chloramphenicol treatment could be the result of proteasome-mediated degradation if this protein was translocated into the host cytosol. Indeed, inhibition of the host proteasome with MG132 prevented degradation of secreted AnkF in the chloramphenicol-treated cells (Fig. 1E), indicating secreted AnkF was located in the host cytosol. Thus, multiple *C. burnetii* Ank proteins are substrates of the Dot/Icm system and AnkF is delivered into host cells during *C. burnetii* infection.

The four translocated *L. pneumophila* Anks were fused to green fluorescent protein (GFP) and ectopically produced in mammalian cells to address whether these proteins possess effector functions. Each of the four Ank proteins showed a different pattern of subcellular localization in mammalian cells, suggesting that these proteins have different targets and distinct functions (Fig. 2A). Extensive fragmentation of the Golgi apparatus was observed in cells producing GFP-AnkX, which correlated with a significant defect in the release of secreted alkaline phosphatase (AP) into the tissue culture medium (Fig. 2C,D). GFP-AnkX deletion derivatives revealed that ARHDs and the amino terminal region of AnkX were both required for disrupting secretory transport (Fig. 2C,D).

Effectors translocated by the Dot/Icm system have predicted roles in blocking fusion of the *L. pneumophila*-containing vacuole with late endosomes and promoting vacuole fusion with endoplasmic reticulum (ER)-derived vesicles (17). To determine whether AnkX is an effector that controls membrane transport, we further investigated the process of AnkX-mediated dispersion of the Golgi apparatus in mammalian cells by time-lapse microscopy. AnkX protein was microinjected into cells producing the resident Golgi enzyme N-acetylgalactosaminyltransferase-II fused to yellow fluorescent protein (GalNAcTII-YFP). Compared to control cells treated with Brefeldin A (BFA, Movie S1), which stimulated retrograde transport of Golgi membranes and cargo back to the ER, microinjected AnkX protein resulted in GalNAcTII-YFP being dispersed in peripheral vesicles formed during Golgi fragmentation, with no apparent delivery back to the ER (Fig. 3A and Movie S2). Thus, the AnkX activity more closely mimicked fragmentation of the Golgi that occurs during mitosis (Movie S2, cells marked "M"), when transport of vesicles from the ER to the Golgi is blocked.

The CopI and CopII coat proteins control initial events important for ER vesicle transport to the Golgi apparatus and colocalize on early secretory vesicles (18). CopI and CopII staining revealed no defects in the morphology of vesicles at ER exit sites in AnkX-injected cells (Fig. 3B), suggesting that AnkX does not affect this early stage in vesicle production from the ER. Interfering with vesicle production from ER exit sites by production of the GDP-locked Arf1 T31N protein blocked AP secretion and reduced *L. pneumophila* replication in mammalian cells (Fig. 3C). AnkX production blocked AP secretion but did not interfere with *L.*

pneumophila replication (Fig. 3C). Thus, AnkX disrupts secretory transport after vesicles have exited the ER.

To address whether AnkX specifically prevented the transport of early secretory vesicles, endocytic transport of transferrin was followed in cells microinjected with AnkX protein (Movie S3). In neighboring cells that were not injected with AnkX, fluorescently-labeled transferrin was internalized and transported to perinuclear sorting compartments (Fig. 3D). In AnkX-injected cells, however, transferrin remained in peripheral endosomes that enlarged over time (Movie S3, Fig. 3D). The effects of AnkX resembled what occurs when cells are treated with the drug nocodazole (19–21), which depolymerizes microtubules. However, tubulin staining in cells microinjected with AnkX revealed no morphological defects in microtubules (Fig. 3E), indicating that AnkX interfered with microtubule-dependent transport of vesicles without affecting the organization of the microtubule network.

Because microtubule-dependent transport is important for early to late endosome maturation (19–21), trafficking of wild type *L. pneumophila* was compared to isogenic $\Delta dotA$ and $\Delta ankX$ mutants to determine if AnkX was important for *L. pneumophila* evasion of endocytic maturation. The majority of vacuoles containing wild type *L. pneumophila* evaded fusion with LAMP-1-positive late endosomes at 30min and 2hr post-infection, whereas, vacuoles containing the *L. pneumophila* $\Delta dotA$ mutant acquired LAMP-1 within the first 30min of infection (Fig. 4A). Most vacuoles containing the *L. pneumophila* $\Delta ankX$ mutant were LAMP-1 negative at 30min (Fig. 4A), however, there was a significant increase at 2hr in the number of LAMP-1-positive vacuoles containing the $\Delta ankX$ mutant. When remodeling of the *L. pneumophila*-containing vacuole by ER-derived vesicles was prevented by treating cells with BFA, there was a significant increase in the number of LAMP-1-positive vacuoles containing *L. pneumophila* $\Delta ankX$ mutants compared to wild type at 30min post-infection (Fig. 4B). The defect in avoiding LAMP-1 acquisition for vacuoles containing the $\Delta ankX$ mutant was suppressed when the microtubule network was destabilized by nocodazole treatment (Fig. 4C, D). By contrast, vacuoles containing the *L. pneumophila* $\Delta dotA$ mutant acquired LAMP-1 rapidly in nocodazole-treated cells. Thus, microtubule depolymerization had a specific effect that restored the ability of the $\Delta ankX$ mutant to efficiently avoid endocytic maturation, indicating that interfering with microtubule-dependent vesicle transport mimics a virulence activity mediated by the AnkX protein during infection.

The *L. pneumophila* and *C. burnetii* Ank proteins represent a large and diverse family of proteins containing ARHDs that we determined to be translocated into eukaryotic cells by pathogen-associated TFSSs. Similar to eukaryotic proteins, the prediction is that these bacterial proteins will have diverse cellular functions that are achieved by targeting different host factors through ARHD-dependent interactions. Finding that the *L. pneumophila* AnkX protein interferes with microtubule-dependent vesicular transport demonstrates that Ank proteins have effector functions. In addition to preventing endocytic maturation of the pathogen-occupied vacuole, AnkX interfered with transport of ER-derived vesicles generated at peripheral ER exit sites, an activity that will facilitate recruitment of early secretory vesicles to the vacuole and aid in construction of the specialized organelle that supports *L. pneumophila* replication (Fig. S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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22. Authors contribution: X.P., A.L., A.S., and M.L. performed research; A.L. conducted studies on the *C. burnetii* Anks; A.S. conducted the AnkX microinjection studies; X.P., A.L., A.S. and C.R. analysed results and wrote the manuscript. We thank G. Warren, F. Gorelick, R. Heinzen, and J. Samuel for reagents. Supported by National Institutes of Health grants AI041699 and AI064559 to C.R., AG030101 and GM060919 to A.S., and Brown-Coxe and Deutsche Forschungsgemeinschaft postdoctoral fellowships to A.L.

Fig. 1.

Type IV translocation of bacterial Ank proteins. (**A,B**) Translocation of Cya-Ank fusion proteins from *L. pneumophila* (A) or *C. burnetii* (B) into CHO-FcγRII cells was determined. Each bar represents the mean cAMP concentration $\pm$ SD from three independent wells. (**C**) RT-PCR analysis of RNA isolated from *C. burnetii*-infected CHO-FcγRII cells. The reverse transcription reaction was omitted in the control lanes to test for DNA contamination. (**D**) Identification of AnkF and EF-Ts in the indicated fractions isolated from human foreskin fibroblasts that were uninfected (–) or infected (+) with *C. burnetii* and either incubated with chloramphenicol (CM) or left untreated. Similar results were obtained from two independent experiments. (**E**) Identification of secreted AnkF in soluble fractions from human foreskin fibroblasts infected with *C. burnetii* that were incubated with chloramphenicol either in the presence or absence of the proteasome inhibitor MG132 as indicated for either 1hr or 2hr. Controls include untreated cells infected with *C. burnetii*, uninfected cells, and actin immunoblots to measure protein loading. Similar results were obtained from two independent experiments.

Fig. 2.

The *L. pneumophila* AnkX protein is an effector of membrane transport. (A) Micrographs indicate the differential localization of *L. pneumophila* Ank proteins fused to GFP in CHO FcγRII cells. (B) Giantin staining (red) in CHO FcγRII cells producing the indicated *L. pneumophila* GFP-Ank proteins (green) reveals that GFP-AnkX production results in disruption of the Golgi apparatus. (C) Amino acid positions (below) and the location of four predicted ARHDs (A1–A4) are indicated in the schematic representation of the AnkX protein. (D) Secretion of alkaline phosphatase (AP) by CHO FcγRII cells producing GFP or the AnkX proteins indicated was measured. The AP index represents the ratio of secreted AP to the amount of AP that remained cell associated. Data are the average ± SD from three independent

samples. Asterisks indicate the samples where the secretion of AP was significantly lower than from GFP-producing cells in an unpaired Student's t-test.

Fig. 3.

AnkX interferes with minus end-directed transport of vesicles on microtubules. **(A)** GalNAcT2-YFP localization in cells treated with Brefeldin-A or injected with purified AnkX. **(B)** CopI and CopII localization in NRK cells injected with purified AnkX (asterix) indicate that Golgi disruption does not affect the recruitment of both coat proteins to ER exit site vesicles. **(C)** CHO Fc γ R2 cells were transfected with plasmids encoding the proteins indicated. Top graph reveals that AnkX production and Arf1T31N production inhibited AP secretion to similar levels. Bottom graph reveals that AnkX production does not affect the efficiency by which *L. pneumophila* forms vacuoles that support replication in CHO Fc γ R2 cells but production of Arf1T31N does. Data are the average $\pm$ SD from three independent samples. **(D)** Micrograph

of Alexa 488-labeled transferrin in cells. The arrow indicates the location of a cell injected with AnkX. **(E)** GalNAc-T2-YFP (green) localization and microtubules (anti- α tubulin, blue) in cells microinjected with purified AnkX (asterix).

Fig. 4.

AnkX prevents microtubule-dependent endocytic maturation of vacuoles containing *L. pneumophila*. (A) Mouse bone marrow-derived macrophages were infected with either wild type *L. pneumophila* (blue), the *dotA* mutant (green), or the *ankX* mutant (pink) for 0.5hr or 2hr as indicated and the percentage of LAMP-1-positive vacuoles for each was determined. Data are the average $\pm$ SD from three independent experiments where at least 50 different vacuoles in different cells were assessed. Asterisks represent data that showed significant difference from control (wild type, 0.5hr) in an unpaired Student's t-test. (B-D) The same assay described in (A) was conducted to examine the effects resulting from disrupting secretory transport using BFA (B), disrupting microtubules using nocodazole (C), or addition of both BFA and nocodazole (D).