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***Vibrio* VopQ Induces PI3-Kinase Independent Autophagy and Antagonizes Phagocytosis**

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Abstract

Vibrio parahaemolyticus (*V. para*) is a Gram-negative bacterium responsible for gastroenteritis acquired from the consumption of contaminated shellfish. This bacterium harbors two type III secretion systems, one on each chromosome. The type III secretion system on chromosome I induces cell death by a temporally controlled sequence of events that is caspase independent and first involves induction of autophagy, followed by cellular rounding, and finally cellular lysis. VopQ is a type III secreted effector that is necessary for the induction of autophagy as mutant strains lacking VopQ are attenuated in their ability to induce autophagy during infection. VopQ is sufficient to induce rapid autophagy as demonstrated by microinjection of recombinant VopQ into GFP-LC3 HeLa cells. Our results demonstrate that VopQ is both necessary and sufficient for induction of autophagy during *V. para*-mediated cell death and this effect is independent of phosphatidylinositol-3-kinases but requires Atg5. Furthermore, induction of VopQ-mediated autophagy prevents recruitment of the necessary cellular machinery required for phagocytosis of *V. para* during infection. These data provide important insights into the mechanism used by *V. para* to cause disease.

Keywords

Autophagy; Phagocytosis; Type III Secretion; *Vibrio parahaemolyticus*

Introduction

Vibrio parahaemolyticus (*V. para*) is a halophilic bacterium commonly found in marine and estuarine environments (Daniels *et al.*, 2000). Infections with *V. para* arise from the consumption of raw or undercooked shellfish and typically result in gastroenteritis. Wound infections can result from handling contaminated products. In rare cases, when the infection becomes systemic, it can be life threatening to individuals with pre-existing health conditions or to those that are immune-compromised (Morris, 2003, Daniels *et al.*, 2000, Nair *et al.*, 2007). *V. para* is endemic to Southeast Asia where consumption of seafood is high. However, this bacterium has become an increasing health concern as isolates have been detected along the western and eastern U.S. seaboards as well as the Gulf Coast (Daniels *et al.*, 2000, Morris, 2003). *V. para* proliferates in warmer temperature waters and consequently, shellfish harbor high bacterial titers in summer months (Kaneko & Colwell, 1975, Colwell, 1973, Kaysner *et al.*, 1990, DePaola *et al.*, 1990). As global warming causes

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ocean temperatures to rise, *V. para* gains access to marine and estuarine environments previously inaccessible due to colder temperatures, thus creating new niches for habitation and colonization (Shope, 1991, McLaughlin *et al.*, 2005).

The most well studied virulence factors of *V. para* are the thermostable direct hemolysin (TDH) and the TDH-related hemolysin (TRH) (Park *et al.*, 2004, Miyamoto *et al.*, 1969, Fukui *et al.*, 2005). However, strains in which the TDH and TRH are deleted are still cytotoxic in tissue culture models of infection (Park *et al.*, 2004, Liverman *et al.*, 2007). Sequencing of the *V. para* genome revealed the presence of two type III secretions systems (T3SS), one on chromosome I (T3SS1), and the other on chromosome II (T3SS2). T3SS1 is required for cytotoxicity in a tissue culture model of infection whereas T3SS2 has been associated with enterotoxicity in the rabbit ileal loop model of infection (Park *et al.*, 2004). Recent results from our lab demonstrate that T3SS1-mediated cytotoxicity is caspase independent and involves induction of autophagy followed by cell rounding and subsequent cell lysis (Burdette *et al.*, 2008). These results would appear to be in conflict with those reported by Ono and colleagues. This group proposed *V. para*-induced cell death was due to apoptosis as measured by annexin V and propidium iodide staining. However, the cell permeability we observe at 3 to 4 hours post infection allows access of the fluorescent annexin V to stain internal phosphatidylserine, thus labeling non-apoptotic cells as apoptotic (Ono *et al.*, 2006, Burdette *et al.*, 2009, Burdette *et al.*, 2008). The form of cell death observed for *V. para* is the first example of the induction and usurpation of autophagy by the T3SS of an extracellular pathogen and therefore research into understanding this mechanism is valuable for understanding *V. para* pathogenesis as a whole.

T3SS1 resembles the T3SS of *Yersinia* spp. in structure and organization except for the addition of unknown genes (*vp1680*, *vp1683*, and *vp1686*) that encode for putative type-III effectors (VopQ, VopR, and VopS, respectively) and predicted cognate chaperones (Ono *et al.*, 2006, Panina *et al.*, 2005, Park *et al.*, 2004). Previous studies from our lab identified autophagy, cell rounding, and lysis as attributes of T3SS1-mediated cell death (Burdette *et al.*, 2008). Disruption of the actin cytoskeleton, which leads to cell rounding was correlated with the presence of VopS, one of the type III effectors secreted from T3SS1 (Casselli *et al.*, 2008). Subsequently, VopS was shown to posttranslationally modify Rho-family GTPases with adenosine monophosphate (AMP). This modification, termed AMPylation, prevents the interaction of Rho-family GTPases with downstream signaling molecules, impairing actin assembly and resulting in cell rounding (Yarbrough *et al.*, 2009).

V. para strains lacking VopS are still cytotoxic indicating that another T3SS1-encoded effector may be responsible for mediating these effects during infection (Yarbrough *et al.*, 2009). Herein, we analyze the role of VopQ in T3SS1-mediated cell death and show that this protein is necessary and sufficient for T3SS1-mediated autophagy. VopQ induces autophagy during infection in a PI3-kinase-independent manner. Finally, electron microscopy analysis shows that *POR3ΔvopQ* infected cells have a high number of intracellular bacteria supporting our hypothesis that the induction of autophagy is affecting the ability of the infected cell to induce the phagocytic machinery. To our knowledge, VopQ is the first bacterial type III effector from an extracellular pathogen identified to induce autophagy. We aim to not only understand the molecular mechanism of VopQ in the context of *V. para* pathogenesis but exploit its usefulness as a tool in furthering our understanding of autophagy during infection.

Results

VopQ is a type III secreted effector essential for T3SS1-mediated cytotoxicity

V. para has previously been shown to secrete several effectors from T3SS1. Among these effectors is VopQ, a 54 kDa protein hypothesized to be involved in cytotoxicity elicited during a tissue culture of infection (Ono *et al.*, 2006). BLAST analysis of the primary amino acid sequence reveals VopQ shares little homology to other bacterial or eukaryotic proteins of known function. However, several orthologues exist in related species (data not shown). To understand the role of VopQ during *V. para* infection and in T3SS1-mediated cytotoxicity, we evaluated *V. para* strains POR1, POR2 and POR3 for the secretion of VopQ in the culture supernatant. Using antibodies against VopQ, we detected VopQ in the culture supernatant of the POR1 and POR3 strains (Figure 1A, lanes 1 and 3), but not in the culture supernatant of the POR2 strain (Figure 1A, lane 2). These results are consistent with previous results demonstrating VopQ is an effector secreted by T3SS1 of *V. para*. Using the *V. para* POR3 strain, we generated an isogenic mutant of the T3SS1-encoded effector *vopQ*. Using antibodies against VopQ, we detected VopQ in the bacterial pellet and culture supernatant of the POR3 strain and the complemented POR3 Δ *vopQ* strain (POR3 Δ *vopQ* + VopQ) (Figure 1B, lanes 1 and 3). However, VopQ is absent in the bacterial pellet and culture supernatant of the POR3 Δ *vopQ* strain (Figure 1B, lane 2). Infection of HeLa cells with *V. para* strains capable of secreting only from T3SS1 (POR3) results in cellular rounding by 2 hours and ultimate lysis by 3 hours compared to mock infected cells (Figure 2, compare G–L with mock infected cells, A–F). However, HeLa cells infected with the POR3 Δ *vopQ* strain show a slight delay in cell rounding (Figure 2, compare panel O with I and U). This phenotype is restored in the complemented strain (Figure 2, S–X). We speculate that other effector(s) may be contributing to the residual cell lysis seen during infection with the POR3 Δ *vopQ* strain. However, not all effectors have been identified for T3SS1.

These phenotypic observations implicate VopQ in the induction of cytotoxicity. To test this, we used an LDH release assay to measure the ability of the POR3, POR3 Δ *vopQ*, POR3 Δ *vopS*, and POR3 Δ *vopQ* + VopQ strains to induce lysis of HeLa cells over an 8 hour time course of infection. Consistent with previous results, POR3 induced rapid cellular lysis, peaking at 4 hours post infection (Figure 2Y, squares). However, the POR3 Δ *vopQ* strain was severely attenuated in its ability to induce lysis. The POR3 Δ *vopQ* infected cells did ultimately release cellular contents, albeit at much later time points (Figure 2Y, right-side up triangles). Deletion of VopS, another T3SS1-encoded effector, does not abrogate the ability of T3SS1 to induce cell lysis (Figure 2Y, upside-down triangles). The complemented strain (POR3 Δ *vopQ* + VopQ) was identical to POR3 in its ability to induce cytotoxicity (Figure 2Y, diamonds). As demonstrated by an attachment assay, each strain is able to make contact with HeLa cells during infection to the same degree (Figure S1). These results demonstrate that VopQ is a type III secreted effector that contributes to T3SS1-mediated cytotoxicity in a tissue culture model of infection.

VopQ is necessary and sufficient to induce autophagy

Infection with *V. para* causes the rapid induction of autophagy that is mediated by T3SS1 (Burdette *et al.*, 2008). Autophagy is the process by which cells undergo bulk degradation of cytosolic contents under starvation conditions for the purpose of providing the cell with nutrients. Membranes form around cytosolic contents, including subcellular organelles, forming autophagosomes. These autophagosomes are targeted to, and ultimately fuse with, lysosomes where resident proteases digest the contents. Nutrients are recycled back into the cell to fend off starvation (Mizushima *et al.*, 2008). This highly regulated process can be monitored both by biochemistry and microscopy (Klionsky *et al.*, 2007). Microtubule-

associated protein light chain 3 (LC3) is a marker of autophagy that, under normal conditions, resides in the cytosol (LC3-I). Upon induction of autophagy, LC3-I is processed and conjugated to phosphatidylethanolamine (PE), forming LC3-II that now associates with autophagosomal membranes (Kabeya *et al.*, 2000, Levine & Yuan, 2005). The GFP-tagged PE-conjugated form of LC3 (GFP-LC3-II) appears by fluorescence microscopy as punctate dots corresponding to forming autophagosomes during starvation. LC3-II also migrates faster than LC3-I on SDS-PAGE gels. Using a HeLa cell line stably expressing GFP-LC3, we can follow induction of autophagy by conversion of GFP-LC3-I to GFP-LC3-II by Western blot analysis and the formation of GFP-LC3 punctae via fluorescence microscopy (Kabeya *et al.*, 2000).

To elucidate whether VopQ plays a role in the induction of autophagy, we infected GFP-LC3 HeLa cells with various strains of *V. para*. Consistent with previous observations, we see an increase in LC3-II formation in the positive control (starved cells) or cells infected with POR3 (Figure 3A, lane 2 and 3, respectively, and Figure 3B) (Burdette *et al.*, 2008). By contrast, infection of GFP-LC3 HeLa cells with the POR3 Δ vopQ strain did not result in conversion of LC3-I to LC3-II (Figure 3A, lane 4, and Figure 3B). An absence of LC3-II formation correlates to reduced GFP-LC3 punctae in POR3 Δ vopQ infected cells compared to starved or POR3 infected cells (Figure 3, compare I to E and G). The addition of protease inhibitors does not change the profile of T3SS1-induced GFP-LC3-II conversion during infection (data not shown). GFP-LC3 punctae levels are similar to that of mock infected cells up to 5 hours post infection in POR3 Δ vopQ-infected cells. (Figure S2). These results show that T3SS1-dependent autophagy requires the type III effector VopQ.

In order to demonstrate that VopQ alone is sufficient to induce autophagy, GFP-LC3 HeLa cells were microinjected with recombinant purified VopQ or GST as a control. Texas-Red conjugated dextran was co-injected to identify the injected cells. Following injection, these marked cells were monitored for the appearance of GFP-LC3 punctae. In control injected cells, the GFP-LC3 signal remained diffuse and cytoplasmic (Figure 4, A and B). However, VopQ injection showed GFP-LC3 punctae after 30 minutes (Figure 4, A and B). Cells injected with VopQ ultimately go on to die at approximately 2 hours post injection (data not shown). To analyze whether these GFP-LC3 punctae were induced via the classic PI3-kinase dependent autophagy pathway, GFP-LC3 HeLa cells were pretreated with the PI3-kinase inhibitors 3-methyladenine (3-MA) (Figure 4, E and F) or wortmannin (Figure 4, G and H). These inhibitors are competent in their ability to inhibit induction of autophagy by rapamycin, a TOR kinase inhibitor and well-characterized inducer of autophagy (Figure S3). The VopQ-mediated GFP-LC3 punctae formation appears to be independent of PI3-kinase activation because treatment with these inhibitors had no effect on the ability of VopQ to induce punctae formation (Figure 4, E-H). Based on our molecular microbiology studies with the POR3 Δ vopQ deletion strain and our cell biology studies with purified recombinant protein, we propose that VopQ is both necessary and sufficient to induce autophagy during *V. para* infection.

T3SS1-mediated induction of autophagy requires the Atg5-Atg12 conjugation system

LC3-I is converted to LC3-II by the addition of a phosphatidylethanolamine group via a ubiquitin-like conjugation system (Kabeya *et al.*, 2000). A second ubiquitin-like system conjugates Atg5 to Atg12 and is required for the modification of LC3. LC3-II cannot be detected in mouse cells deficient for Atg5 (Tanida *et al.*, 2004). In order to ascertain the contribution of Atg5 to T3SS1-mediated autophagy, we tested *atg5*^{-/-} MEFs for the ability of T3SS1 to induce LC3 conversion. Consistent with previous results in multiple cell types, POR3 infected cells induced robust endogenous LC3-II conversion compared to mock infected cells (Figure 5, lane 2). As expected, in the absence of Atg5, we see no LC3-II conversion during POR3 infection (Figure 5, lane 4). These results confirm that the LC3-II

conversion seen during infection involves classical autophagic machinery and suggests T3SS1 induces autophagy upstream of the LC3 and Atg5-Atg12 conjugation systems.

VopQ induces autophagy independent of PI3-kinases

Incubation of GFP-LC3 HeLa cells with the PI3-kinase inhibitors 3-MA or wortmannin prior to microinjection does not abrogate punctae formation (Figure 4, E–H). In addition, despite the fact that treatment of GFP-LC3 cells with wortmannin during infection abrogates GFP-LC3 punctae formation, some punctae remain (Burdette *et al.*, 2008). This suggests that during *V. para* infection, autophagy is proceeding by both a PI3-kinase-dependent (wortmannin-sensitive) and PI3-kinase-independent (wortmannin-insensitive) pathway. We hypothesize that VopQ is inducing autophagy via a PI3-kinase-independent mechanism. To address this, we infected GFP-LC3 HeLa cells with POR3 or POR3 Δ vopQ in the absence or presence of wortmannin treatment (Figure 6). Wortmannin-treated POR3-infected cells or untreated POR3 Δ vopQ-infected cells show less punctae accumulation relative to untreated POR3-infected cells consistent with previous data (Figure 6, F–H or I–K compared to C–E) (Burdette *et al.*, 2008). In contrast, wortmannin treatment combined with infection with POR3 Δ vopQ strains almost completely abrogates GFP-LC3 punctae formation (Figure 6, L–N). Analysis of GFP-LC3-II conversion by Western blot supports our microscopic observations (Figure 6A and 6B). These results are consistent with our hypothesis that VopQ is inducing autophagy independent of PI3-kinases. LPS-mediated signaling through Toll-like receptors may ultimately be responsible for GFP-LC3-II conversion in all strains at later timepoints (Delgado *et al.*, 2008)

Induction of autophagy by VopQ prevents phagocytosis

We have observed autophagic vesicles in both HeLa cells and macrophages during infection with *V. para* (Burdette *et al.*, 2008). We hypothesized that induction of rapid autophagy may be redirecting the cellular machinery essential for phagocytosis. Therefore, we examined POR3 and POR3 Δ vopQ-infected macrophages for the presence of intracellular bacteria by electron microscopy. POR3-infected macrophages had few, if any, intracellular bacteria at 1 and 3 hours post infection with extracellular bacteria surrounding lysed cells (Figure 7A and 7B). In contrast, macrophages infected with the POR3 Δ vopQ strain show the presence of intracellular bacteria at both 1 and 3 hours post infection (Figure 7C and 7D). At 3 hours post infection, host cells are observed that have greater than 4 intracellular bacteria per cell (Figure 7E). These results demonstrate that the presence of the type III effector VopQ attenuates phagocytosis of *V. para* during infection.

Discussion

V. para uses a temporally controlled mechanism to induce cell death that involves autophagy, modulation of the actin cytoskeleton and finally, cell lysis (Burdette *et al.*, 2008). In this manuscript, we characterize the type III effector VopQ as a key mediator of autophagy and cell lysis. Consistent with previous observations, we show that VopQ is secreted by T3SS1 (Ono *et al.*, 2006). We demonstrate herein that *V. para* strains lacking VopQ are attenuated in their ability to induce cytotoxicity in a tissue culture model of infection. Cells infected with the POR3 Δ vopQ strain did not round up as fast as cells infected with POR3 (Figure 2; compare panel O with I and U). In addition, cells infected with POR3 release cellular contents by 3 hours post infection while cells infected with the POR3 Δ vopQ strain are intact up to 5 hours post infection. This demonstrates that VopQ is a key virulence factor in T3SS1-mediated cytotoxicity.

V. para can be counted among the bacterial and viral pathogens that hijack the autophagy pathway for their own benefit (Colombo, 2007). Herein, we showed that the *V. para* T3SS1

effector VopQ is essential for autophagy during infection because the *POR3ΔvopQ* strain is attenuated in its ability to induce autophagy as measured by GFP-LC3-II conversion and GFP-LC3 punctae formation. Due to the severe toxicity seen during transfection of cells with VopQ, we turned to a recombinant protein microinjection system and demonstrate that VopQ is sufficient for the induction of autophagy. This system is analogous to the type III secretion system, except that the amounts of protein and refolding requirements are distinct.

In previous studies, we were able to reduce the number of punctae observed in *POR3*-infected cells with the PI3-kinase inhibitor wortmannin (an inhibitor of class I and class III PI3-kinases) (Powis *et al.*, 1994, Burdette *et al.*, 2008). We were unable to use the PI3-kinase inhibitor 3-MA (an inhibitor specific for class III PI3-kinase) during infection as 3-MA is toxic to *V. para* (data not shown) (Seglen & Gordon, 1982). Interestingly, when we pretreated cells with either inhibitor, we were unable to prevent GFP-LC3 punctae formation in cells microinjected with recombinant VopQ. These observations support the hypothesis that VopQ is inducing autophagy in a PI3-kinase-independent manner but requires the Atg5-Atg12 conjugation system.

We are currently pursuing the nature of the PI3-kinase-independent, VopQ-dependent induction of autophagy. What are the roles of autophagy during infection? Induction of autophagy may be the bacteria's way of forcing the cell to provide nutrients for the bacteria in a readily usable form. By inducing autophagy, the bacteria manipulate the host cell into degrading intracellular proteins and organelles, generating essential amino acids and cofactors. Upon lysis, these degraded components are liberated for consumption by the pathogen. In addition, we speculate that induction of autophagy may be a way to prevent phagocytosis of an extracellular pathogen. We examined *POR3* infected cells for the presence of intracellular bacteria and found that although electron microscopic images of *V. para* infected cells showed the appearance of early autophagosomal structures, these cells also lacked intracellular bacteria. Images of cells infected with the *POR3ΔvopQ* strain lacked signs of autophagosomal structures, yet an overwhelming number of cells contained intracellular bacteria. We propose that induction of autophagy sequesters the necessary membrane components that are required for phagocytosis. In fact, many of the cellular factors involved in autophagic vesicle nucleation and transport are also required for generating sufficient membranes to engulf bacteria during phagocytosis (Fader & Colombo, 2009, Xie & Klionsky, 2007, Yoshimori & Noda, 2008). These cellular factors can include PI3-kinase signaling and the actin cytoskeleton (Reggiori *et al.*, 2005, Monastyrska *et al.*, 2008, Stephens *et al.*, 2002). Proteins required for trafficking of multivesicular bodies (MVB) (Rab5, Rab7, and ESCRT complexes) are also found on autophagosomal membranes and are involved in phagocytosis (Deretic & Fratti, 1999, Rusten & Simonsen, 2008, Deretic, 2008). Herein, we present a novel mechanism used by the type III effector VopQ from the extracellular pathogen *V. para* to manipulate the host cellular response to infection. Further research will not only aid in our understanding of the pathogen but also identify critical cellular factors that govern the host cells' decision between life and death.

Experimental Procedures

Strains and growth media

V. para strains are passaged on minimal marine media containing galactose as a carbon source (MMM) without antibiotics (*POR3* and *POR3ΔvopQ*) or with tetracycline (20 μg ml⁻¹) (*POR3ΔvopQ* + VopQ) or on MMM + sodium pyruvate where indicated. Liquid cultures are grown in LB + 3% NaCl (MLB) at 30°C unless otherwise specified. To induce the *V. para* T3SS, overnight cultures are diluted 1:50 into fresh MLB with 10 mM MgCl₂ and 10 mM sodium oxalate and grown at 37°C for 3 hours.

Cell lines

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 M L-glutamine, 100 $\mu\text{g ml}^{-1}$ streptomycin, 100 U ml^{-1} penicillin, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate (Invitrogen) at 37°C with 5% CO_2 . The GFP-LC3 HeLa cell lines were maintained as described but with 100 $\mu\text{g ml}^{-1}$ G418. Immortalized wild-type (*atg5*^{+/+}) and Atg5 deficient (*atg5*^{-/-}) murine embryonic fibroblasts (MEFs) were maintained in 50% DMEM and 50% F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 $\mu\text{g ml}^{-1}$ streptomycin, 100 U ml^{-1} penicillin, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate (Invitrogen) and 142 μM β -mercaptoethanol. The wild-type MEFs, *atg5*^{-/-} MEFs, and FP-LC3 stable HeLa cell line were generous gifts from Beth Levine and generated as described (Kuma *et al.*, 2004, Kabeya *et al.*, 2000). RAW 264.7 macrophages were cultured in DMEM with 10% FBS (Sigma), non-essential amino acids (Invitrogen) and 10 mM L-glutamine (Invitrogen).

Generation of Knockout Strains

vopQ and 1 kb of flanking upstream and downstream genomic regions was cloned into pLafR (Boles & McCarter, 2000) to yield pLafR.*vopQ* (tetracycline^R). Primers harboring 50 nucleotides of homologous sequences to the regions flanking VopQ were used to amplify the chloramphenicol cassette from pKD3, which was subsequently used for lambda red recombination (Datsenko & Wanner, 2000). The resultant pLafR.*vopQ*::Cm^R was conjugated into *V. para* via tri-parental mating and transconjugants were selected on MMM with tetracycline (20 $\mu\text{g ml}^{-1}$). Following selection on tetracycline, bacteria were passaged on heart infusion (HI) with chloramphenicol (10 $\mu\text{g ml}^{-1}$) to select for recombination of the cassette. To resolve merodiploids, the plasmid pPH1JI (gentamicin^R) was used to kickout the knockout plasmid via incompatibility through selection on MMM + gentamicin (100 $\mu\text{g ml}^{-1}$). The resulting clones were verified for sensitivity to tetracycline, and resistance to chloramphenicol and gentamicin. Disruption of *vopQ* was verified by polymerase chain reaction and sequencing. Absence of VopQ secretion was verified by *in vitro* secretion assays. The pPH1JI plasmid was cured by passaging *V. para* Δ *vopQ* strains on MLB agar at 37°C three times for three days. Complemented strains were obtained by conjugating the pLafR.*vopQ* plasmid via tri-parental mating and selecting for tetracycline resistance.

In vitro secretion assays

Bacteria were grown as described above to induce secretion. Following induction, 1 O.D.₆₀₀ of bacteria were harvested via centrifugation. The pellet was resuspended in SDS sample buffer and boiled for 10 minutes. The supernatant was filtered with a 0.2 μm syringe filter and TCA was added to a final volume of 20% and incubated at 4°C overnight. BSA (6 μg) was added as a TCA precipitation control. TCA-precipitated supernatants were centrifuged at 20,000 $\times g$ for 10 minutes at 4°C and washed with ice-cold acetone. Pellets were resuspended in SDS sample buffer and boiled for 10 minutes.

Infections

All infections were performed in culture media in the absence of antibiotics. HeLa cells or RAW macrophages were seeded at a density of 0.15×10^6 or 0.5×10^6 cells per ml respectively into 6 well dishes. For experiments involving MEFs, cells were seeded at a density of 0.05×10^6 cells per ml. After 18–24 hours, bacteria were added at an MOI of 10. The plates were centrifuged at 200 $\times g$ for 5 minutes. At indicated time points, cells were harvested for Western blot analysis by the addition of SDS sample buffer. For confocal microscopy, cells were plated as above on sterile glass coverslips and at indicated time points fixed in 3.2% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature.

Preparation of slides for confocal microscopy

Following fixation, cells were permeabilized in 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Cells were stained with rhodamine-phalloidin (1:40, Molecular Probes) and Hoechst (1:10000, Sigma) in PBS plus 0.1% BSA for 20 minutes at room temperature. Following 2 washes in PBS, slides were mounted with 10% glycerol containing n-propyl galate and sealed with clear nail polish. Samples were visualized with a Zeiss LSM 510 scanning confocal microscope. Images were converted using ImageJ software and Adobe Photoshop.

Measurement of LDH Release

HeLa cells were infected as described over an 8 hour time course. Cells were either left untreated, infected with POR3, POR3 Δ vopQ, or POR3 Δ vopQ + VopQ. LDH release was measured with a Cytotoxicity Detection kit (Takara). Results are expressed as cytotoxicity calculated as the percent of total lysis in 1% Triton X-100.

Bacterial Attachment Assay

HeLa cells were plated for infections as described above. Cells were infected with POR3, POR3 Δ vopQ, POR3 Δ vopS, and POR3 Δ vopQ + VopQ. At 30 minutes post infection, HeLa cells were gently washed three times in PBS and incubated in 0.1% Triton X-100 for 15 minutes at room temperature to lyse HeLa cells. This lysate was diluted serially and plated onto MMM + sodium pyruvate to determine the number of bacteria attached. Bacterial input was also plated onto MMM + sodium pyruvate. Percent attachment is expressed as the percentage of input bacteria recovered following infection.

Methods for Monitoring Autophagy

To induce autophagy, GFP-LC3 HeLa cells were starved in Hank's Balanced Salt Solution (Invitrogen) with protease inhibitors pepstatin A (10 μ g ml⁻¹, Sigma) and E64-d (10 μ g ml⁻¹, Sigma) for four hours. Cells were harvested in SDS sample buffer, boiled for 5 minutes and samples were either immediately processed for Western blotting or frozen at -20°C. GFP-LC3-I and GFP-LC3-II were detected using an anti-GFP antibody (JL-8, Molecular Probes). Endogenous LC3 was detected in wild-type and *atg5*^{-/-} MEFs using anti-LC3 antibody (Novus). The Atg5-Atg12 conjugate was detected using an anti-Atg5 antibody (Novus). Identical samples were probed with an anti-actin antibody (Sigma) to confirm equal loading. Relative LC3-II accumulation was determined by quantitating band intensity using ImageJ software and calculating the ratio of LC3-II to LC3-I.

Preparation of Samples for Electron Microscopy

Samples were prepared with the assistance of Laurie Mueller at the Molecular and Cellular Imaging Core Facility at the University of Texas Southwestern Medical Center. RAW 264.7 macrophages were infected as described. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. After solvent dehydration, centrifuged pellets of cells were embedded in epoxy resin (EMBED 812, Electron Microscopy Sciences) and polymerized at 60°C. Ultrathin sections were cut at a nominal thickness of 80 nm, picked up on copper grids, and stained with uranyl acetate and lead citrate. Sections were examined at 120 kV with a Tecnai G² Spirit transmission electron microscope (FEI Company), and images were acquired with a Soft Imaging System Morada camera. Cells were counted in a blinded fashion for the presence of intracellular bacteria. For every 100 cells counted, cells were scored for the presence of intracellular bacteria and the number of intracellular bacteria per cell. Cells were counted 3 times for each sample.

Purification of recombinant VopQ

The coding region corresponding to *vopQ* was cloned into pET15b (ampicillin^R, Novagen) at the NdeI and BamHI restriction sites to generate pET15b.*vopQ*. This construct was transformed into competent *E. coli* BL21-DE3 (Novagen) and cultures were grown to an O.D.₆₀₀ of 0.6–0.8 at 37°C and induced with 0.4mM IPTG for 4 hours at 25°C. Bacterial pellets were lysed in phosphate buffered saline with 1% Triton X-100 and VopQ was purified by Ni²⁺-affinity chromatography (Qiagen).

Microinjection of recombinant purified VopQ

Microinjection was performed with a Transjector 5246 and a Micromanipulator 5171 (Eppendorf) as described previously (Bartz *et al.*, 2008). HeLa cells stably expressing GFP-LC3 were grown over night on glass coverslips and injected into the cytoplasm with 0.5 mg ml⁻¹ VopQ or 0.5 mg ml⁻¹ GST as a control. Prior to injection, the proteins were dialyzed against 25 mM HEPES-KOH pH 7.4, 50 mM KAc and mixed with 2 mg ml⁻¹ lysine fixable 70 kDa Texas-Red dextran (Invitrogen) as an injection marker. After the injections, the cells were incubated for 30 min at 37°C and then fixed. For inhibitor experiments with VopQ, cells were pretreated for 45 minutes with 10 μM wortmannin or 10 mM 3-MA. For the rapamycin experiment, cells were pretreated for 30 minutes with 10μM wortmannin or 10 mM 3-MA and then treated with 1 μg ml⁻¹ rapamycin for 4 hours. All inhibitors were present throughout the duration of all experiments. The cells were fixed for 15 min in 3.7% formaldehyde in PBS and permeabilized for 10 minutes in methanol at -20° C. DNA was then stained with Hoechst 33342 (Invitrogen) and the cells were mounted in Mowiol 4-88 (Calbiochem). Fluorescence analysis was performed with an Axiovert 200M microscope (Zeiss) and a LD Plan-Neofluar 40x/1.3 DIC objective (Zeiss). Images were captured with an Orca-285 camera (Hamamatsu Photonics) and the software package Openlab 4.02 (Improvision).

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Figure 1.

VopQ is an effector secreted by *V. para* T3SS1. **(A)** Overnight cultures of *V. para* POR1, POR2 and POR3 were diluted back into secretion inducing media and grown for 3 hours at 37°C. Anti-VopQ antibody was used to probe TCA-precipitated culture supernatants for the secretion (TCA) of VopQ. The membrane of TCA-precipitated supernatants was stained with coomassie blue for BSA as a loading control (BSA). The data are representative of three independent experiments. **(B)** Overnight cultures of POR3, POR3ΔvopQ, and POR3ΔvopQ + VopQ strains were treated as in (A) and anti-VopQ antibody was used to probe bacterial pellets and TCA-precipitated culture supernatants for the production (Pell) and secretion (TCA) of VopQ as in (A). The membrane of TCA-precipitated supernatants was stained with coomassie blue for BSA as a loading control (BSA). The data are representative of three independent experiments.

Figure 2.

T3SS1 cytotoxicity is attenuated in the absence of VopQ. (A–X) HeLa cell monolayers were either mock infected (A–F) or infected with *V. para* strains POR3 (G–L), POR3ΔvopQ (M–R), and POR3ΔvopQ + VopQ (S–X). At the indicated timepoints, cells were fixed and processed for confocal microscopy with rhodamine phalloidin to stain for actin (red) and Hoechst for nuclei (blue). Scale bar represents 10 μm. The data are representative of three independent experiments. (Y) HeLa cells were infected as described above. At the indicated timepoints, culture supernatants were evaluated for the release of LDH as a measure of cytotoxicity and reflected as percent of total cellular lysis. The data are the means ± SD from a representative experiment repeated in triplicate. Mock (●), POR3 (■), POR3 ΔvopQ (▲), POR3ΔvopS (▼), and POR3 ΔvopQ + VopQ (◆)

Figure 3.

VopQ is necessary for T3SS1-mediated induction of autophagy. **(A)** GFP-LC3 HeLa cells were either mock infected (Lane 1), starved with protease inhibitors (lane 2), or infected with POR3, POR3ΔvopQ, and POR3ΔvopQ + VopQ (lanes 3, 4, and 5) for 2 hours and lysates were probed with anti-GFP and anti-actin antibodies. The data are representative of three independent experiments. **(B)** Relative LC3-II accumulation was determined as described in Experimental Procedures. The data are the means ± SD from three independent experiments. **(C–L)** GFP-LC3 HeLa cells were infected as described in (A) and processed for confocal microscopy with staining with Hoechst for nuclei (blue), rhodamine phalloidin for actin (red) as described in Experimental Procedures and visualized for GFP-LC3 punctae formation (green). Scale bar represents 10 μm. The data are representative of three independent experiments.

Figure 4.

VopQ is sufficient for T3SS1-mediated induction of autophagy. GFP-LC3 HeLa cells were microinjected with either recombinant 0.5 mg ml^{-1} GST (control) or rHis₆-VopQ (VopQ) along with Texas-Red dextran as an injection marker. Cells were either left untreated (**A–D**) or preincubated with PI3-kinase inhibitors 3-MA (10 mM) (**E–F**) and wortmannin (10 μM) (**G–H**) for 30 minutes prior to injection and throughout the duration of the experiment. Cells were fixed at 30 minutes post injection and DNA was stained with Hoechst. Red, Texas-Red dextran injection marker; blue, DNA; green, GFP-LC3. Scale bar represents 15 μm . The data are representative of three independent experiments.

Figure 5.

T3SS1-mediated induction of autophagy requires the Atg5-Atg12 conjugation system. Wild-type or *atg5*^{-/-} murine embryonic fibroblasts were either mock-infected or infected with POR3. At 1.5 hours post infection, cells were lysed in SDS sample buffer and evaluated for LC3 conversion by Western blot using an anti-LC3 antibody and for the presence of the Atg5-Atg12 conjugate using an anti-Atg5 antibody. The membrane used to detect the Atg5-Atg12 conjugate was re-probed with anti-actin to confirm equal loading. The data are representative of three independent experiments.

Figure 6.

VopQ induces autophagy independent of PI3-kinases. **(A)** GFP-LC3 HeLa cells were treated with wortmannin for 30 minutes prior to infection and all cells were either infected with POR3, POR3 in the presence of wortmannin (10 μ M), POR3 Δ vopQ, or POR3 Δ vopQ in the presence of wortmannin (10 μ M). Samples were processed for Western blot analysis at the indicated timepoints and probed with an anti-GFP antibody. The data are representative of three independent experiments. **(B)** Relative LC3-II accumulation was determined as described in Experimental Procedures. The data are the means $\pm$ SD from a representative experiment repeated in triplicate. **(C–N)** Cells were infected as above and processed for confocal microscopy with staining with Hoechst for nuclei (blue) as described in Experimental Procedures and visualized for GFP-LC3 punctae formation (green). Scale bar represents 10 μ m. The data are representative of three independent experiments.

Figure 7.

Induction of autophagy prevents phagocytosis. (A–D) RAW 264.7 macrophages were infected with POR3 or POR3 $\Delta vopQ$. At the indicated timepoints, cells were fixed and processed for electron microscopy as described in Experimental Procedures. Black arrows indicate autophagic vesicles. White arrows indicate intracellular bacteria. Scale bars represent 2 μ m. The data are representative of three independent experiments. (E) Cells were scored for the presence of intracellular bacteria and the number of bacteria per cell is reflected as described in Experimental Procedures. The data represent the means $\pm$ SD from a representative experiment.