Inhibition of MAPK Signaling Pathways by VopA from Vibrio parahaemolyticus*

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During infection, bacterial pathogens utilize a type III secretion system to inject effectors into the cytoplasm of a target cell where they disrupt the defense system of the host cell. Vibrio parahaemolyticus, a causative agent of gastroenteritis endemic in Southeast Asia, has a type III secretion system that encodes a novel member of the YopJ-like protein effector family, VopA (Vibrio outer protein A). Our studies revealed that Vibrio VopA encodes an evolutionarily conserved activity that is extremely potent and requires an intact catalytic site to abrogate signaling pathways in a manner distinct from that of other YopJ-like effectors. We observed that VopA efficiently inhibits the MAPK signaling pathways but not the NFκB pathway in mammalian cells. When expressed in yeast, VopA induces a growth arrest phenotype and also blocks yeast MAPK signaling pathways. Our observations provide insight into the immense diversity of targets utilized by YopJ-like effectors to manipulate eukaryotic signaling machineries that are important for the response and survival of the host cell during infection and/or symbiosis.

The Gram-negative marine bacterium Vibrio parahaemolyticus is a major agent responsible for gastroenteritis outbreaks associated with the consumption of infected seafood (1). Areas of recent outbreaks range from the coastal regions of North America to Eastern Asia. The bacterial pathogen Yersinia pestis is the causal agent for the bubonic plague, whereas Yersinia pseudotuberculosis and Yersinia enterocolitica are causal agents for gastrointestinal disorders (2). Salmonella typhimurium is a facultative intracellular pathogen that causes gastroenteritis, and because it is carried by a variety of animal hosts, it is of increasing concern to the food industry in the developed world (3). All three pathogens harbor pathogenicity islands that encode a type III secretion system, which is used to inject effector proteins into the host cytoplasm. Effector proteins are molecules that mimic a eukaryotic activity and are used by pathogens to disrupt signaling in the infected cell. All three of the aforementioned bacterial pathogens encode an effector protein that belongs to the family of YopJ-like proteins.

YopJ, the founding member of this family, is an effector protein expressed by Yersinia spp. that is able to block the innate immune response in infected target cells (4). Expression of cytokines and anti-apoptotic factors are prevented during a Yersinia infection because YopJ is able to inhibit MAPK signaling pathways and the NFκB pathway by preventing the activation of the superfamily of MAPK kinases (5). Later studies demonstrated that the family of YopJ-like proteins encodes a cysteine protease domain and that the wild type but not the catalytically inactive forms are able to inhibit signaling pathways (6). The family of YopJ-like proteins is found in a wide variety of animal and plant pathogens as well as the plant symbiont Rhizobium (4, 7). The effector AvrA from S. typhimurium is the only other YopJ-like protein from an animal pathogen studied to date and is implicated in the inhibition of the NFκB pathway (8).

V. parahaemolyticus is phylogenetically close to Vibrio cholerae (the causative agent of cholera), and recent sequencing of the V. parahaemolyticus genome reveals the existence of two pathogenicity islands (PAI and PAII), which are not found in V. cholerae (9). Encoded within PAII is a type III secretion system and an effector protein, referred to as VopA, sharing ~55% similarity with the YopJ-like proteins from Yersinia and Salmonella. V. parahaemolyticus is a bacteria that is associated with two hosts: shellfish and human. Within shellfish, it exists as a commensal, whereas in humans, it is an incidental pathogen (acquired by eating contaminated shellfish). YopJ-like effectors are expressed by a number of bacteria, including pathogens, commensals, and symbionts. These effector proteins, like other type III secreted virulence factors, are expressed by the bacteria and injected into the target host cell where they manipulate the host machinery. Our studies reveal that VopA, encoded by V. parahaemolyticus, is able to manipulate eukaryotic signaling machineries, and this may be of use during its existence as a commensal or incidental pathogen. Herein, we observe that VopA functions in a mechanistically similar manner to other YopJ-like proteins but demonstrates a remarkable diversity in its inhibitory profile of signaling pathways.

**EXPERIMENTAL PROCEDURES**

Cloning of Effectors—VopA was amplified by PCR from V. parahaemolyticus genomic DNA with a 5′- HindIII primer and a 3′-FLAG-stop

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s)AY606230 and AY597337.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; HOG, high osmolality growth.
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In NFκB/H9260 luciferase reporter assays, VopA is unable to inhibit TNFα/H9251-induced activity. Taken together, these observations support the hypothesis that VopA has no effect on the NFκB pathway, which is in contrast to the two other YopJ-like effectors from animal pathogens, YopJ and AvrA.

VopA Inhibits the Mammalian MAPK Pathways—Yersinia YopJ is able to inhibit MAPK signaling pathways, so we next assessed the ability of Vibrio VopA to inhibit the mammalian MAPK pathways. 293T cells were transfected with various YopJ-like effectors in the presence of HA-ERK followed by treatment with epidermal growth factor (5). Both wild-type YopJ and VopA, but not the catalytically inactive forms of these effectors (YopJC172A or VopAC167A), are able to inhibit extracellular growth factor-induced ERK activation (Fig. 2A). In a similar fashion, both YopJ and VopA are able to inhibit the other MAPK pathways. These studies provide the first evidence that Vibrio VopA is able to inhibit mammalian signaling pathways. However, the various YopJ-like effectors appear to target distinct sets of signaling pathways within the eukaryotic cell, because in contrast to YopJ, no interaction is observed between VopA and the family of MAPK kinases in yeast two-hybrid binding assays.

Both YopJ and VopA are very efficient at inhibiting the MAPK pathways, but detecting the expression of these YopJ-like proteins is extremely difficult (Fig. 2A). To determine the potency of inhibition by these effectors, we analyzed their MAPK inhibitory activity over a range of expression conditions. Inhibition of the MAPK signaling pathway is observed with as little as 1 ng of YopJ- or VopA-transfected DNA. Although the inhibition of the MAPK pathways is comparable between the two effectors, the amount of YopJ-FLAG protein appears to be at least 10-fold greater than that of VopA-FLAG (as detected by anti-FLAG immunoblots) (Fig. 2B).

The Role of VopA in Programmed Cell Death—Having established a profile of inhibitory activity for these YopJ-like effectors, we wanted to investigate what role these effectors (independent of other pathogenic factors) play on the fate of an infected cell. We utilized the well established system in

**Fig. 4. Vibrio VopA inhibits growth when expressed in yeast.** Growth assay demonstrates the effect of effector expression on growth in liquid media containing glucose (Glu) (A), galactose (Gal) (B), and galactose with 1 m sorbitol (Gal+Sorb) (C). D, growth assay demonstrates the effect of effector expression on yeast growth on media containing glucose, galactose, and galactose with 1 m sorbitol. Data shown are representative of three experiments.

**Fig. 5. Vibrio VopA inhibits MAPK signaling when expressed in yeast.** The ability of YopJ and VopA to inhibit activation of the yeast MAPKs Hog1p and Mpk1p is assayed by inducing cells with either 0.7 mM sorbitol for 2.5 min (A) or 12 mM caffeine for 1 h (B). C, control vector; J, YopJ; JC/A, YopJ C172A; V, VopA; VC/A, VopA C167A; MKK, MAPK kinase; MKKK, MAPK kinase kinase; IKK, IkB kinase β.
Expression of VopA in Yeast Causes a Growth Arrest Phenotype—One of the characteristics of bacterial pathogenic effectors secreted by a type III secretion system is that their catalytic activity of the effector and its mutant counterpart, under the control of a galactose-inducible promoter, were grown to mid-log phase in glucose media followed by plating onto glucose or galactose media. When yeast cells containing VopA plasmid are plated on galactose media containing 1 M sorbitol, the expression of anti-apoptotic factors (11, 12). Effectors were transfected into Jurkat cells and following stimulation with TNF-α, were assessed for survival (Fig. 3). Cell death was not observed in control cells in the presence or absence of stimulus. Wild-type YopJ, but not the catalytically inactive form of YopJ, is able to efficiently promote cell death in Jurkat cells but only in the presence of stimulus, which supports the hypothesis that the Yersinia YopJ effector does not directly activate death machinery but promotes cell death by the blocking of signaling pathways. The ability of VopA to promote cell death in the presence of stimulus is marginal (Fig. 3) and cannot be attributed to blocking the NF-kB pathway but may result as an indirect consequence of its extremely potent inhibition of the MAPK pathways.

Inhibition of MAPK Pathways by VopA Is Evolutionarily Conserved—To investigate whether VopA, like YopJ, inhibits MAPK signaling pathways in yeast, cells containing the various effectors were plated on galactose media with 1 M sorbitol, which results in the activation of the NF-kB pathway (HOG) MAPK pathway (10). As expected, yeast expressing either YopJ or VopA, but not the catalytically inactive mutants, plated on galactose media containing 1 M sorbitol are unable to grow (Fig. 4D). Consistent with our observations in mammalian systems, Salmonella AvrA shows no obvious inhibitory affect on the MAPK pathways in yeast (data not shown). When yeast expressing YopJ or VopA are grown in liquid media containing 1 M sorbitol, the growth phenotype is quite striking (Fig. 4C). Growth is almost immediately arrested in the presence of VopA, whereas in the presence of YopJ, the growth arrest requires more time. These observations further emphasize the difference in targets between YopJ and VopA and indicate that the growth arrest phenotype in the presence of VopA does not depend only on the inhibition of the activated HOG MAPK pathway.

To test the ability of VopA to inhibit the HOG MAPK pathway, yeast cells were grown in glucose media to mid-log phase and then transferred to galactose media to induce expression of the YopJ-like proteins. These cells were incubated with sorbitol, and the lysates of cells were analyzed for induction of the HOG MAPK pathway (10). Yeast cells containing either a control vector or the plasmids encoding catalytically inactive forms of YopJlike proteins. Yeast cells containing a control vector or vector expressing YopJ, YopJ C172A, or VopA C167A grow at approximately the same rate (Fig. 4B). In contrast, the growth of VopA-expressing yeast cells is dramatically hindered, but they continue to grow, albeit at a slower rate than the other strains (Fig. 4B). In cells expressing VopA, growth arrest is observed over an extended period of time, indicating that the growth arrest is not likely to be a cell cycle-dependent defect. The phenotype is dependent on wild-type VopA and not the catalytically inactive form of VopA, indicating that the mechanism of inhibition is similar to other YopJ-like effectors but that the targets are distinct.

Fig. 6. A, model depicting the various inhibitory activities of Yersinia YopJ, Vibrio VopA, and Salmonella AvrA on mammalian signaling pathways is shown. B, alignment of the catalytic core of VopA, YopJ, and AvrA is shown. Asterisks designate catalytic residues.
of YopJ or VopA are able to activate Hog1p via phosphorylation, whereas wild-type YopJ and VopA inhibit Hog1p activation (Fig. 5A). Similar results are observed using caffeine to induce the cell wall integrity MAPK pathway (14); yeast cells expressing wild-type YopJ or VopA, but not their mutant counterparts, are unable to induce the activation of Mpk1p via phosphorylation in this pathway (Fig. 5B). VopA, like YopJ, inhibits evolutionarily conserved MAPK signaling pathways in yeast, but unlike YopJ, demonstrates the ability to induce a growth arrest phenotype in yeast indicating a distinct set of targets for VopA.

The profiles of inhibition for each of the mammalian YopJ-like effectors are clearly distinct. VopA potently blocks the evolutionarily conserved MAPK pathways, YopJ blocks MAPK pathways and the NFκB pathway (5), and AvrA blocks only the NFκB pathway (8) (Fig. 6A). Each pathogen has evolved to encode a YopJ-like effector displaying a unique inhibitory activity that requires a common catalytic site (Fig. 6B) that benefits its respective pathogen in different ways. The activity of Yersinia YopJ complements its fellow effectors by blocking the production of cytokines and anti-apoptotic machinery, thereby preventing the induction of the immune response and promoting apoptosis. Salmonella AvrA does not affect MAPK signaling in mammalian cells or yeast but does inhibit the NFκB pathway (8), albeit weakly, and does abrogate the constitutive degradation of β-catenin (15). This inhibitory activity results in c-Myc-induced proliferation of the infected cell, which may prolong the life of the infected cell and allow for propagation of an intracellular pathogen (15). Vibrio VopA, which we have shown to be an extremely potent inhibitor of the evolutionarily conserved MAPK pathways, may function to inhibit cytokine production resulting in attenuation of the host defense response. The activity of VopA along with other virulence factors could facilitate colonization in the intestine of humans resulting in propagation of the pathogen. Alternatively, VopA may be used to abrogate signaling while existing as a commensal in the shellfish host. Currently, only laborious animal models (e.g. the ligated rabbit ileal loop model) exist for studying V. parahaemolyticus (16), but systems to study infection by V. parahaemolyticus using a tissue culture-based approach are currently being developed.

In summary, three different types of intestinal pathogens have usurped a structurally conserved and mechanistically similar activity in the form of a YopJ-like effector. Recent studies suggest that this activity was originally captured by the plant bacterial pathogen, Xanthomonas, which expresses not only YopJ-like effectors (encoding remote similarity to a ubiquitin-like protein protease (Ulp1)) but also the effector XopD (a member of the Ulp1 protein family) (4, 17). XopD is a constitutively active enzyme with plant-specific deSUMOylating enzyme activity (17). These observations support a hypothesis that pathogenic bacteria have usurped the Ulp1 hydrolase activity. The catalytic domain of this molecule may then have been transformed into a unique activity that is maintained and used by a specific pathogen for targeting and inhibiting a distinct set of signaling pathways for the advantage of the pathogen during infection of the host. As YopJ effectors are maintained and expressed by both plant and animal pathogens as well as a plant symbiont (and most likely in yet to be discovered animal commensals), they provide powerful tools for identifying critical steps in eukaryotic signaling machineries that are important for host response and survival during infection and/or symbiosis.

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