Identification of a Molecular Target for the *Yersinia* Protein Kinase A

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SUMMARY

Pathogenic bacteria of the genus *Yersinia* employ a type III secretion system to inject bacterial effector proteins directly into the host cytosol. One of these effectors, the *Yersinia* serine/threonine protein kinase YpkA, is an essential virulence determinant involved in host actin cytoskeletal rearrangements and in inhibition of phagocytosis. Here we report that YpkA inhibits multiple Gα signaling pathways. The kinase activity of YpkA is required for Gα inhibition. YpkA phosphorylates Ser47, a key residue located in the highly conserved diphosphate binding loop of the GTPase fold of Gα. YpkA-mediated phosphorylation of Ser47 impairs guanine nucleotide binding by Gα. *Y. pseudotuberculosis* expressing wild-type YpkA, but not a catalytically inactive YpkA mutant, interferes with Gα-mediated signaling pathways. Identification of a YpkA-mediated phosphorylation site in Gα sheds light on the contribution of the kinase activity of YpkA to *Yersinia* pathogenesis.

INTRODUCTION

The genus *Yersinia* includes three species of Gram-negative bacteria that are pathogenic for humans: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. pestis* is of worldwide concern, being responsible for the bubonic plague, also known as the Black Death. This organism has had catastrophic effects on the population of this planet, killing more than 200 million people during the middle ages (Perry and Fetherston, 1997). The ability of *Yersinia* spp. to cause disease is in part due to the presence of an extrachromosomal 70 kb virulence plasmid, referred to as the *Yersinia* outer protein (Yop) virulon (Cornelis et al., 1998). The Yop virulon encodes a variety of bacterial virulence factors and a type III protein secretion system (T3SS; Cornelis and Van Gijsenem [2000]). The T3SS is a sophisticated translocation apparatus highly conserved among many Gram-negative pathogens that is used to deliver bacterially encoded proteins directly into the host cytosol. T3SSs are present in animal, plant, and insect pathogens, underscoring the widespread use of this secretion system and the significance of the virulence mechanism (Galan and Collmer, 1999). *Yersinia* uses the T3SS to deliver at least six different Yop effector proteins into the host cell: YopH, a protein tyrosine phosphatase; YpkA (called YopO in *Y. enterocolitica*), a protein kinase; YopT, a cysteine protease; YopJ (called YopP in *Y. enterocolitica*), an acetyltransferase; YopE, a GTPase-activating protein; and YopM, a leucine-rich protein of unknown cellular function (Cornelis, 2002a, 2002b; Mukherjee et al., 2006; Navarro et al., 2005; Viboud and Bliska, 2005). Once inside the host cell, the concerted activities of the Yop effectors participate in thwarting the host antimicrobial defenses and in “hijacking” normal cellular signaling pathways, thus promoting survival and replication of the bacteria (Aepfelbacher, 2004; Navarro et al., 2005; Viboud and Bliska, 2005).

The *Yersinia* protein kinase A is essential for *Yersinia* virulence and shares sequence homology in its amino terminus with eukaryotic serine/threonine kinases (Galyov et al., 1993). *Y. pseudotuberculosis* mutant strains expressing catalytically inactive YpkA variants are markedly attenuated in virulence in mouse infection studies (Galyov et al., 1993). In cell culture infection assays, the enzymatic activity of YpkA was necessary for inhibition of host cell bacterial internalization (Grosdent et al., 2002; Trasak et al., 2006; Wiley et al., 2006). Both the N-terminal kinase domain and the C-terminal Rho-GTPase binding domain act synergistically to disrupt the host cell actin cytoskeleton (Dukuzumuremyi et al., 2000; Hakansson et al., 1996; Juris et al., 2000). The crystal structure of the C-terminal Rho-GTPase binding domain of YpkA has recently been solved (Prehna et al., 2006). Several key features of YpkA suggest that it might interfere with host cell signaling. First, activation of its kinase activity is dependent on the YpkA-mediated phosphorylation of Ser47, a key residue located in the highly conserved diphosphate binding loop of the GTPase fold of Gα. YpkA phosphorylates Ser47, a key residue located in the highly conserved diphosphate binding loop of the GTPase fold of Gα. YpkA-mediated phosphorylation of Ser47 impairs guanine nucleotide binding by Gα. *Y. pseudotuberculosis* expressing wild-type YpkA, but not a catalytically inactive YpkA mutant, interferes with Gα-mediated signaling pathways. Identification of a YpkA-mediated phosphorylation site in Gα sheds light on the contribution of the kinase activity of YpkA to *Yersinia* pathogenesis.
of YpkA localizes the protein to the inner surface of the host cell plasma membrane, where it is in close proximity to key proteins involved in transducing extracellular signals into eukaryotic cells (Dukuzumuremyi et al., 2000; Hakansson et al., 1996; Viboud and Bliska, 2005). Third, it is striking that YpkA has sequence identity to the eukaryotic kinases and that key residues involved in catalysis have been maintained over billions of years of evolution (Galyov et al., 1993). These observations suggest that the kinase activity of YpkA plays an important role in its function as a virulence factor.

Heterotrimeric G proteins couple with G protein-coupled receptors (GPCRs) to transduce signals from a myriad of extracellular agents and play a central regulatory role in a number of cellular activities, such as cell growth and proliferation, neuronal signaling, hematopoietic cell differentiation, leukocyte activation, platelet aggregation, glucose secretion, actin cytoskeletal reorganization, and ion channel regulation (Offermanns, 2003; Wetttschreck and Offermanns, 2005). These proteins are composed of three subunits: the \( \alpha \) subunit has GTPase activity and contains the guanine-nucleotide binding site, while the \( \beta \) and \( \gamma \) subunits interact to form a tight dimer. Upon agonist binding, the GPCR adopts an active conformation that facilitates the exchange of GDP for GTP on the \( \alpha \) subunit of the G protein, leading to dissociation of the G\( \alpha \) and G\( \beta \gamma \) subunits. The active GTP-bound form of the \( \alpha \) subunit and the G\( \beta \gamma \) dimer subsequently associate with and activate downstream effector proteins involved in critical cellular processes. G proteins are divided into four families based on sequence similarities of the \( \alpha \) subunits: G\( \alpha \), G\( \alpha \)i/o, G\( \alpha \)12/13, and G\( \alpha \)q. Members of the G\( \alpha \)s and G\( \alpha \)i families are known to activate and inhibit adenylyl cyclase, respectively. Members of the G\( \alpha \)s/12/13 family regulate the small G protein RhoA, while G\( \alpha \)q family members stimulate phospholipase C-\( \beta \) (PLC-\( \beta \)), leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG). In addition, G\( \alpha \)q family members have also been shown to activate RhoA-mediated pathways.

The importance of heterotrimeric G protein \( \alpha \) subunits in eukaryotic defense responses is underscored by the observation that a number of bacterial pathogens have evolved toxins that specifically target their activity. For example, *Vibrio cholerae* and *Bordetella pertussis* secrete toxins that interfere with G\( \alpha \)s and G\( \alpha \)i/o signaling, respectively (Krueger and Barbieri, 1995). In these cases, ADP ribosylation of the \( \alpha \) subunits contributes to disease progression.

In this study, we investigated the cellular function and molecular target(s) of the kinase domain of YpkA during pathogenesis. We show that YpkA inhibits GTP binding by the heterotrimeric G protein, G\( \alpha \)q. The mechanism involved a YpkA-mediated phosphorylation event on a critical serine residue on G\( \alpha \)q required for guanine nucleotide binding. These findings further our knowledge of the molecular mechanism used by YpkA during *Yersinia* pathogenesis and suggest a role for G\( \alpha \)q in host antimicrobial defenses.

**RESULTS**

**The Kinase Activity of YpkA Is Necessary for Inhibition of G\( \alpha \)q Signaling Pathways**

YpkA is a multidomain protein (Figure 1A). The N-terminal serine/threonine kinase domain is preceded by a region mediating YpkA secretion and translocation and is followed by a Rho GTPase binding GDI-like domain. A stretch of amino acids at the very C terminus of YpkA are involved in actin binding and subsequent autoactivation of YpkA (Juris et al., 2000). YpkA utilizes both kinase-dependent and kinase-independent mechanisms to disrupt the host actin cytoskeleton (Prehna et al., 2006; Wiley et al., 2006). The C terminus of YpkA binds to the small Rho GTPases, RhoA and Rac1, and has recently been shown to possess guanidine nucleotide dissociation inhibitor (GDI) activity (Prehna et al., 2006). However, the cellular function and molecular target(s) of the kinase domain are unknown.

Previous reports have alluded to a role for the small G protein RhoA in YpkA-mediated cytoskeletal rearrangements (Barz et al., 2000; Dukuzumuremyi et al., 2000). In addition, YpkA has been shown to reduce the level of GTP-bound RhoA in *Yersinia*-infected cells (Barz et al., 2000; Dukuzumuremyi et al., 2000). To further explore this observation and to assign a role to either the N-terminal kinase domain or the C-terminal GDI domain, we first determined the minimal C-terminal fragment of YpkA required to cause disruption of the actin cytoskeleton. Successive N-terminal truncations of YpkA resulted in a fragment of YpkA containing amino acids 399–729, YpkA\( 399-729 \), that was sufficient for inhibition of stress fiber formation, in agreement with Prehna et al. (2006) (Figure 1B and data not shown). Next, we assayed the ability of wild-type YpkA or YpkA\( 399-729 \) to inhibit actin stress fiber assembly mediated by the constitutively active mutant of RhoA, RhoA\( G14V \). Surprisingly, RhoA\( G14V \) induced actin stress fiber assembly in transfected HEK293A cells in the presence of either full-length YpkA or the C-terminal fragment containing the GDI activity, suggesting that YpkA may be exerting its inhibition on the actin cytoskeleton upstream of the small GTPase RhoA (Figures 1C and 1D).

Activation of GPCRs leads to the induction of actin cytoskeletal rearrangements by activating signaling pathways that involve RhoA (Sah et al., 2000). Most of the GPCR agonists that regulate Rho-dependent cytoskeletal responses couple to the heterotrimeric G proteins, G\( \alpha \)12/13 and G\( \alpha \)q. We assessed the ability of wild-type YpkA to inhibit actin stress fiber assembly induced by a subset of GPCR agonists known to activate RhoA by diverse signaling pathways (Sah et al., 2000). For these experiments, we used the GPCR agonists, carbachol, thrombin, and lysophosphatidic acid (LPA), which function primarily through G\( \alpha \)q, G\( \alpha \)12, and G\( \alpha \)13, respectively. Carbachol stimulation of HEK293A cells stably expressing the muscarinic M1 receptor led to formation of new actin stress fibers (Figure 2A). Interestingly, transfection of wild-type YpkA,
but not a catalytically inactive variant of YpkA, YpkA_{D267A}, abrogated carbachol-induced actin reorganization (Figures 2A and 2B). The YpkA_{D267A} mutant containing the intact GDI domain also had no effect on the ability of carbachol to stimulate actin stress fibers in HEK293A cells (Figures 2A and 2B). Thus, this inhibition was a result of YpkA kinase activity and not due to the GDI activity of YpkA. HEK293A cells labeled with an anti-vinculin antibody confirmed disruption of stress fibers by YpkA (see Figure S1 in the Supplemental Data available with this article online). YpkA also inhibited both thrombin- and LPA-induced actin stress fiber assembly. However, this inhibition was not due to the kinase activity of YpkA (see the Supplemental Data text and Figure S2).
Figure 2. YpkA Inhibits Gαq-Mediated Actin Stress Fiber Formation in a Kinase-Dependent Manner
(A) Immunofluorescence microscopy of carbachol-stimulated HEK293A cells transfected with GFP, GFP-YpkA, GFP-YpkA_{D267A}, or GFP-YpkA_{399–729}. Merged image of YpkA (green) and actin (red) in the left panel and actin morphology is shown in the right panel.
(B) Quantification of actin stress fiber assembly in HEK293A cells transfected with plasmids shown in (A) and either treated with carbachol (black bars) or left untreated (gray). One hundred fifty transfected cells were scored in at least three independent experiments. Data are presented as mean ± SEM.
(C) Signaling pathway illustrating carbachol-induced activation of the muscarinic M1 receptor resulting in actin stress fiber formation via a Gαq/LARG RhoGEF/RhoA pathway.
(D) Quantification of actin stress fiber assembly in HEK293A cells transfected with 0.5 μg of either GFP, GFP-YpkA, or GFP-YpkA_{D267A} and 3-fold excess (1.5 μg) of vector, RhoA_{G14V}, LARG RhoGEF, or Gαq_{Q209L}. One hundred fifty transfected cells were counted in three independent experiments. Data are presented as mean ± SEM.
Upon binding to the muscarinic M1 receptor, carbachol promotes Gaq-coupled activation of RhoA via the leukemia-associated Rho guanine nucleotide exchange factor (LARG RhoGEF) (Booden et al., 2002; Reuther et al., 2001). We conducted a series of epistasis experiments to identify the molecular target(s) of YpkA. As shown in Figure 2D, expression of GaqG14V and LARG RhoGEF suppressed YpkA cytotoxicity. Interestingly, YpkA abolished stress fiber formation induced by a constitutively active mutant of Gaq, GaqQ209L, and pronounced retraction fibers were present in these cells, a hallmark of YpkA cytotoxicity (Figure 2D and Figure S3). To further explore this observation, we used the kinase-inactive mutant of YpkA to evaluate the role of the kinase domain of YpkA. A catalytically competent form of YpkA was required to block Gaq-mediated actin stress fiber formation (Figure 2D). In control experiments, GaqG14V, LARG RhoGEF, and GaqQ209L induced actin reorganization as previously reported (Figure 2D) (Sah et al., 2000). These data suggest that the kinase activity of YpkA is necessary for inhibition of Gaq signaling pathways.

YpkA Phosphorylates Gaq in Intact Cells and Binds to Gaq via Its Kinase Domain

We performed metabolic labeling experiments to assess Gaq phosphorylation by YpkA in vivo. HEK293A cells were transiently transfected with GaqQ209L in the presence of either vector, wild-type YpkA or YpkAD267A. Following a 2 hr incubation with [32P]orthophosphate, Gaq was immunoprecipitated from cell lysates with a specific anti-Gaq antibody. Phosphorylated proteins were separated on an SDS gel and visualized by autoradiography. As shown in Figure 3A, incorporation of [32P]orthophosphate into the GaqQ209L protein was observed in the presence of the catalytically active YpkA kinase, but not in vector controls or upon exposure of equal amounts of the inactive kinase-dead YpkAD267A. These data indicate that YpkA functions as a kinase for Gaq when expressed in intact HEK293A cells.

In vivo interactions between exogenous Gaq and YpkA were evaluated by coimmunoprecipitation. Heterotrimeric Ga proteins exist as inactive GDP-bound and active GTP-bound forms. Figure 3B shows that YpkA bound equally to wild-type Gaq and GaqQ209L. Anti-FLAG immunoprecipitations (IP) were probed by EE immunoblot (IB) (upper panel). Cell lysates were probed by EE or FLAG immunoblot to show input levels (lower two panels).

Mapping of the YpkA Phosphorylation Site(s) on Gaq

We next analyzed the residue(s) phosphorylated in Gaq by YpkA. Lysates (bottom panel), were analyzed by western blotting with anti-GFP and anti-Gaq antibodies.
In vivo phosphorylation of \( \text{G}_\alpha \) was performed as described in Figure 3A in the absence of \([g-32P]ATP\). HEK293A cells were transiently transfected with wild-type YpkA and \( \text{G}_\alpha \text{Q}_{209L} \). \( \text{G}_\alpha \) immunoprecipitated from whole-cell lysates was separated by SDS-PAGE and visualized by Coomassie blue staining. The band corresponding to \( \text{G}_\alpha \) was excised from the gel and subjected to in-gel tryptic digestion, and the resulting peptides were analyzed by nano LC tandem mass spectrometry. One tryptic peptide (872.50 m/z, charge

Figure 4. Analysis of YpkA Phosphorylation Sites on \( \text{G}_\alpha \)

(A) Phosphorylated tryptic peptide from \( \text{G}_\alpha \). \( \text{G}_\alpha \) proteins share a common guanine nucleotide binding domain. The five polypeptide loops, designated G-1 through G-5, that form the guanine nucleotide binding site are the most highly conserved elements in this domain and define the \( \text{G} \) protein superfamily. The conserved nucleotide binding motif in the G-1 box of \( \text{G}_\alpha \) is shown in bold letters. The three potential phosphorylation sites (Ser44, Ser47, and Thr48) are underlined. The phosphorylation site (Ser47) is in red.

(B) Identification of Ser47 of \( \text{G}_\alpha \) as an in vivo YpkA phosphorylation site. In vivo phosphorylation of \( \text{G}_\alpha \text{Q}_{209L} \) was performed as described in Figure 3A in the absence of \([g-32P]ATP\). \( \text{G}_\alpha \) protein bands were excised from the Coomassie-stained gel and digested with trypsin, and the peptides were analyzed by nano LC tandem mass spectrometry. The mass spectra of the tryptic peptide shown was manually analyzed and revealed Ser47 as the phosphorylation site (red).

(C) Ser47 of \( \text{G}_\alpha \) is the major YpkA-catalyzed phosphorylation site in vivo. HEK293A cells cotransfected with \( \text{G}_\alpha \text{WT} \) (lanes 1 and 2), \( \text{G}_\alpha \text{Q}_{209L} \) (lanes 3 and 4), \( \text{G}_\alpha \text{Q}_{209L/S47A} \) (lanes 5 and 6), or \( \text{G}_\alpha \text{Q}_{209L/S44A} \) (lanes 7 and 8) and FLAG-YpkA plasmids were metabolically labeled with \([32P]H_3PO_4\). Following solubilization of the cells, \( \text{G}_\alpha \) was precipitated with an anti-\( \text{G}_\alpha \) antibody. Shown is an autoradiogram of SDS-PAGE (upper panel). Anti-\( \text{G}_\alpha \) immunoprecipitations (IP) were probed by \( \text{G}_\alpha \) (IB) (middle panel). Western blot shows similar proteins were loaded in the experiments. Cell lysates were probed by FLAG immunoblot to show input levels (lower panel).
state 2+) was identified as a phosphorylated peptide from Gαq (LLLLGTGESGKSTFIK, amino acids 36–51) and contained three potential phosphorylation sites: Ser44, Ser47, and Thr48 (Figure 4A). Fragmentation of these peptides revealed that Ser47 is the phosphorylated residue (Figure 4B). To investigate whether or not Ser47 served as an actual YpkA phosphorylation site, we generated the Gαq (S47A) mutant and assessed its phosphorylation by YpkA in [32P]-labeled cells. Mutation of Ser47 to alanine significantly abolished phosphate incorporation into Gαq, indicating that this residue is the predominant site on Gαq phosphorylated by YpkA in vivo (Figure 4C, lane 6). In support of these results, an alanine substitution in an adjacent serine residue, Ser44, resulted in a Gαq partially phosphorylated as GαqS47A (Figure 4C, lane 6). Notably, we observed that YpkA preferentially phosphorylated the active form of Gαq in our metabolic labeling experiments (Figure 4C, lanes 2 and 4). Remarkably, Ser47 is located within the consensus sequence, GXXXXGK (S/T), of the diphosphate binding loop, a very highly conserved region shared by G protein α subunits and critical for guanine nucleotide binding and coordination of the magnesium ion (Figure 4A) (Sprang, 1997).

**Phosphorylation of Gαq by YpkA Decreases Its Ability to Bind GTP**

Since the key event in G protein-mediated signaling is activation of the G protein promoted by the binding of GTP, we wanted to compare the GTP binding properties of unphosphorylated GαqQ209L versus YpkA-phosphorylated GαqQ209L. In order to evaluate the effects of phosphorylation on GTP binding by Gαq, we utilized an assay based upon the binding of [35S]GTPγS to Gαq (Dowling et al., 2004). We generated a mutant Gαq in which Ser47 was replaced with aspartate as a phosphomimetic substitution. GαqQ209L/S47D was expressed as efficiently as wild-type Gαq and GαqQ209L (data not shown). Membranes prepared from transiently transfected HEK293A cells expressing vector, GαqWT, GαqQ209L, or GαqQ209L/S47D, were incubated with [35S]GTPγS and 200 μM carbachol (Dowling et al., 2004). Gαq was subsequently immunoprecipitated with a Gαq-specific antibody, and bound [35S]GTPγS was counted directly. As shown in Figure 5A, we observed that GαqQ209L/S47D mutant, we observed no significant increase in [35S]GTPγS binding (Figure 5A). In control experiments, wild-type Gαq-transfected cells showed a slight increase in GTP binding over vector-transfected cells (Figure 5A). Furthermore, the importance of Ser47 in G protein activation is underscored by the observation that substitution of Ser47 with alanine (GαqQ209L/S47A) also resulted in decreased GTP binding (Figure 5A). These data are in agreement with published results indicating that mutations in homologous residues in other Gα proteins result in diminished GTP binding (Feig and Cooper, 1988; Slepak et al., 1993; Szeberenyi et al., 1990). Thus, our results suggest a mechanism for the inhibition of Gαq by YpkA: namely, covalent modification of Gαq by phosphorylation on a critical serine residue, Ser47, prevents G protein activation.

**Figure 5. Phosphorylation of Gαq by YpkA Impairs Its Ability to Bind GTP and Activate Downstream Signaling Pathways**

(A) Quantification of carbachol-stimulated [35S]GTPγS binding to Gαq and the indicated mutants. Membranes (Dowling et al., 2004) prepared from HEK293A cells transiently transfected with vector, GαqWT, GαqQ209L, GαqQ209L/S47D, or GαqQ209L/S47A were incubated with [35S]GTPγS in the presence of 200 μM carbachol for 30 min at 30°C. Samples were immunoprecipitated with a Gαq-specific antibody, and the amount of bound GTPγS was counted directly. Data are expressed as a percentage (mean ± SEM) of the response in GαqQ209L membranes from four experiments.

(B) Quantification of nuclear translocation of tubby in HEK293A cells cotransfected with either 0.5 μg of GFP-tubby and 3-fold excess (1.5 μg) FLAG-YpkA or FLAG-YpkAΔ267A following carbachol stimulation. One hundred fifty transfected cells were counted in three independent experiments. Data are presented as mean ± SEM.

Heterotrimeric G proteins of the Gq/11 family mediate a wide range of diverse cellular responses, including cell growth and proliferation, neuronal signaling, platelet aggregation, and actin cytoskeleton reorganization (Hubbard and Hepler, 2006). The transcription regulator tubby is involved in maturity-onset obesity in mice and is known to be a downstream target of Gαq. Tubby localizes to molecular targets for YpkA.
Identification of a Molecular Target for YpkA
the plasma membrane by binding phosphatidylinositol
4,5-bisphosphate (Santagata et al., 2001). Receptor-mediated activation of Gαq is thought to release tubby from the plasma membrane through the activity of PLC-β, triggering translocation of tubby to the nucleus (Santagata et al., 2001). We postulated that if YpkA impaired Gαq-nucleotide binding, then all Gαq downstream signaling should also be affected. To test this hypothesis, we analyzed the nuclear translocation of tubby in cells expressing wild-type YpkA and the catalytically inactive YpkA<sub>G276A</sub> mutant after carbachol stimulation. Activation of Gαq by the Gαq-coupled receptor, muscarinic M1, was used to test for translocation inhibition. Figure 5B shows that, in vector-transfected control cells, tubby efficiently translocates to the nucleus. However, in cells expressing wild-type YpkA, we observed a dramatic loss in Gαq-mediated nuclear translocation of tubby. This inhibition was not observed in cells expressing the kinase-inactive YpkA mutant. We confirmed in separate experiments that the S47D mutant of Gαq was deficient in its ability to induce stress fiber formation and the nuclear translocation of tubby (data not shown). Taken together, these results support our previous findings and suggest that the kinase activity of YpkA functions to inhibit multiple downstream Gαq signaling pathways.

**Infection of HEK293A Cells with Yersinia-Expressing YpkA Abolishes Gαq-Mediated Signaling**

We examined the role of YpkA in Gαq signaling during a *Yersinia* infection. We investigated actin stress fiber formation and nuclear translocation of tubby in Gαq<sub>Q209L</sub>-expressing HEK293A cells after infection with *Y. pseudotuberculosis* mutant strains (Dukuzumuremyi et al., 2000). One of these strains, the multiple *yop* mutant (MYM), is deficient in the expression of the *Yersinia* Yop proteins and thus is not cytotoxic to host cells (Figure 6A). In contrast, disruption of the actin cytoskeleton and pronounced retraction fibers were observed in cells infected with the MYM strain expressing either wild-type YpkA (MYM+wtYpkA) or the catalytically inactive YpkA<sub>Q270A</sub> variant of YpkA (MYM+YpkA<sub>Q270A</sub>; see the Experimental Procedures). The ability of inactive YpkA to disrupt the actin cytoskeleton is presumably due to its C-terminal YpkA Rho GDI activity (Dukuzumuremyi et al., 2000). YpkA delivered by the T3SS abolished Gαq-induced stress fiber formation in HEK293A cells, while the YpkA<sub>G276A</sub> variant was unable to prevent the assembly of stress fibers by Gαq<sub>Q209L</sub> (Figure 6B). In control experiments, the MYM host strain had no affect on the ability of Gαq to induce actin stress fiber formation (Figure 6B). As summarized in Figure 6C, these results suggest that, during an infection, *Yersinia*-translocated YpkA functions to disrupt Gαq-Q209L-mediated actin cytoskeletal rearrangements.

We next examined the ability of type III delivery of YpkA to interfere with the nuclear translocation of tubby. HEK293A cells transiently transfected with tubby were subsequently infected with the MYM *Yersinia* strains for 2 hr to ensure delivery of YpkA. Following carbachol stimulation, tubby localized predominantly to the nucleus as confirmed by nuclear staining with Hoechst (Figure 6D). Consistent with our previous findings, the MYM strain had no affect on Gαq signaling in HEK293A cells, since tubby was observed in the nucleus of these cells (Figure 6D). As shown in Figure 6D, nuclear translocation of tubby was essentially blocked by the type III delivery of wild-type YpkA (Figure 6D). In contrast, infection with the MYM+YpkA<sub>Q270A</sub> strain did not prevent the accumulation of tubby in the nucleus after carbachol stimulation (Figure 6D). In control experiments, localization of tubby was similar in cells infected with the MYM *Yersinia* strains as in uninfected cells (data not shown). Collectively, these results strongly suggest that, during an infection, *Yersinia*-translocated YpkA interferes with Gαq signaling (Figure 6E).

**DISCUSSION**

**Gαq: A Molecular Target for YpkA**

YpkA inhibits Gαq signaling by a YpkA-mediated phosphorylation event on Ser47. In its active GTP-bound state, Gαq transduces extracellular signals to downstream effectors. By impairing nucleotide binding by Gαq, YpkA prevents activation of Gαq-mediated cellular responses. We speculate that the presence of a negative charge at Ser47 as a result of phosphorylation induces a conformational change that decreases the affinity of...
Gαq for GTP. Remarkably, Ser47 of Gαq is homologous to the S47C mutant of Gαo and the S17N mutant of p21ras. Both of these mutants display reduced guanine nucleotide binding (Feig and Cooper, 1988; Slepak et al., 1993; Szeberenyi et al., 1990). Furthermore, the S47C mutant of Gαo is attenuated in the G protein-coupled thyreropin-releasing hormone-mediated chloride response (Slepak et al., 1993). In addition, the S17N p21ras mutant inhibited NIH3T3 cell proliferation, and nerve growth factor induced morphological differentiation of PC12 cells (Feig and Cooper, 1988; Slepak et al., 1993; Szeberenyi et al., 1990).

Phosphorylation of G protein α subunits has been reported as a mechanism for regulation of G protein activity (Chen and Manning, 2001). Several Gα subunits are substrates for phosphorylation by protein kinase C and p21-activated protein kinase (Chen and Manning, 2001). Phosphorylation in both instances inhibits the activities of these subunits with the Giβγ heterodimer and regulator of G protein signaling (RGS) proteins and thus might prolong the activation of the Gα subunit. However, phosphorylation has little or no effect on the ability of Gαz-GTPγS to inhibit the effector adenyl cyclase (Kozasa and Gilman, 1996). Regardless of their highly conserved recognition site for guanine nucleotides, not all Gα subunits were phosphorylated in these studies. To our knowledge, we have identified Ser47 of Gαq as a YpkA-mediated phosphorylation site.

Members of the Gαq family of G proteins mediate the activation of PLC-β in response to stimulation of a variety of GPCRs (Hubbard and Hepler, 2006). PLC-β hydrolyzes phosphatidylinositol bisphosphate, generating the second messengers DAG and IP3. DAG activates protein kinase C, and IP3 stimulates the release of Ca2+ from intracellular stores. These metabolic products play critical roles in activation of NADPH oxidase (Minakami and Sumimoto, 2006). Yersinia YpkA may enhance bacterial virulence by inhibiting superoxide generation.

In addition to activation of the classical PLC-β pathway, Gαq has been reported to interact with various candidate effector proteins, regulatory proteins, and a growing list of scaffolding and adaptor proteins (Bhatnagar et al., 2004; Hubbard and Hepler, 2006). Of interest, caveolin is a membrane-bound scaffolding protein that associates with numerous lipid-modified signaling molecules and is enriched in specialized membrane microdomains called caveolae. Given the importance of caveolin-1 in Gαq-coupled receptor signaling and the observation that caveolae-mediated endocytosis is utilized by pathogenic bacteria to gain entry into the host cell, it is not surprising that Yersinia has evolved a mechanism to co-opt Gαq signaling (Bhatnagar et al., 2004). Ric-8A and tubulin stimulate Gαq-mediated signal transduction in a receptor-independent manner (Popova et al., 1997; Popova and Rasenick, 2000; Tall et al., 2003). Ric-8A is a guanine nucleotide exchange factor for Gαq, and tubulin is a cytoskeletal GTP binding protein that activates Gαq via direct transfer of GTP. Inhibitors that interfere with microtubule assembly block invasin-promoted internalization of Yersinia, thus highlighting the importance of the tubulin-Gαq interaction.

A Model for the Action of YpkA during a Yersinia Infection

The detailed molecular mechanism driving YpkA-mediated inhibition of Gαq is currently unclear. We propose that, during a Yersinia infection, host antimicrobial responses lead to the activation of Gαq signaling pathways (Handley et al., 2006; Hoffmann et al., 2004). As a countermeasure, Yersinia injects the Yop proteins (YpkA, YopE, YopT, YopM, YopH, and YopJ) into the host cell. The C-terminal RhoA GDI-like activity of YpkA, in concert with the GTPase activity of YopE and the cysteine protease activity of YopT, mediates disruption of the host actin cytoskeleton by targeting the RhoGTPases and thus contributes to the antiphagocytic ability of Yersinia. Although these bacterial effectors share overlapping virulence functions, they also have unique activities. For example, YopE can inhibit caspase-1-mediated maturation of prointerleukin-1β in macrophages (Schotte et al., 2004; Trasak et al., 2006). Therefore, while the Yops share some level of functional redundancy, they also possess distinct activities.

Indeed, we have identified a function for the kinase domain of YpkA. We observed that YpkA preferentially phosphorylates the active GTP-bound form of Gαq. Phosphorylation of Gαq results in a protein with decreased affinity for GTP. As the number of binding partners for Gαq continues to grow, we can only speculate as to which downstream signaling event(s) is affected by YpkA. In support of our findings, a requirement for the YpkA kinase activity in inhibition of bacterial uptake mediated by the Yersinia adhesin YadA was recently reported (Trasak et al., 2006). Although there is indirect evidence for contribution of Gαq to phagocytosis, a clear role for Gαq has not been established. This observation is intriguing and remains the focus of our research.

Gαq: A Clue to Yersinia Pathogenesis

Why is Gαq targeted by Yersinia YpkA? The most informative clue may come from genetic studies using whole-animal models demonstrating the physiological importance of Gαq. Among the Gαq family members, mice with selective disruption of Gαq have the most pronounced phenotype, indicating that Gαq has unique functions that cannot be complemented by other Gαq family members (Hubbard and Hepler, 2006). Since Gαq is the only Gαq family member expressed in platelets, Gαq knockout mice have increased bleeding times and defective platelet activation, as well as other physiological defects (Offermans et al., 1997). Remarkably, one of the hallmarks of the plague is the bleeding abnormalities which contribute to Yersinia pestis being referred to as the “black death.” It is intriguing to think that YpkA may contribute to this profound phenotype. It is clear that understanding the biological function of essential virulence factors such as YpkA may provide...
strategies for combating the use of Yersinia pestis as a biological weapon.

**EXPERIMENTAL PROCEDURES**

**Plasmids**
The Y. enterocolitica YpkA ORF (YopO) was isolated by PCR using the plasmid pYV80811 (a generous gift from James Bliska, The State University of New York at Stony Brook). Full-length YpkA and its various mutants were cloned in-frame into the pEGFP-C3 (Clontech) and FLAG-tagged pDCNAS.1 (Invitrogen) vectors following standard protocols. RhoA, Gsa, Gs12, Gs13, and muscarinic acetylcholine receptor (M1) cDNAs were purchased from the UMR C DNA Resource Center. p15SRhoGEF was obtained from Dr. Joan Heller Brown. pcDNA5 (LAM) and pcDNA3.1 (Invitrogen) gift of Dr. Channing J. Der. pGFPc1-Tubby was kindly provided by Dr. Lawrence Shapiro. All point mutations were introduced by the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. All constructs were verified by DNA sequencing.

**Cell Culture, Transfection, and Cell Microscopy**
HEK293A cells were maintained as described previously (Juris et al., 2000) and transfected using the FuGENE 6 transfection kit according to the manufacturer’s protocol (Roche Molecular Biochemicals). For all carbachol experiments, we used HEK293A cells stably expressing the M1 muscarinic receptor (M1R) to make these cells responsive to carbachol since they do not express endogenous M1Rs. This cell line was established by transfection of the M1 receptor cDNA (G418 resistant) and isolation of drug-resistant colonies after growth in medium supplemented with G418 (200 µg/ml). Multiple drug-resistant colonies were pooled together to establish the stable cell line. Cell microscopy was performed as previously described (Juris et al., 2000).

**Immunoprecipitation and Western Blot**
After transfection for 16–20 hr, cells were lysed with modified RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P40) containing protease inhibitors (Complete; Roche Molecular Biochemicals). The homogenate was centrifuged (14,000 g, 4°C, 20 min), and the supernatants were incubated with the indicated antibodies bound to protein A beads overnight at 4°C while rotating. After incubation, immunoprecipitates were washed extensively with ice-cold modified RIPA buffer. Proteins bound to the beads were eluted by heating at 95°C for 4 min in SDS-PAGE sample loading buffer. The eluted proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the specified antibodies followed by chemiluminescence detection. The following antibodies were used: anti-EE monoclonal (Covance), anti-FLAG-M2 (Sigma), and anti-Gsa (E-17) (Santa Cruz Biotechnology).

**In Vivo Labeling with [32P]Orthophosphate**
HEK293A cells were cultured and transfected as described above. Twenty four hours after transfection, the cells were washed with phosphate-free DMEM. Following a 2 hr incubation with the same medium containing [32P]Orthophosphate (150 µCi/ml), GsaQ2026A was immunoprecipitated from cell lysates with a specific anti-Gsa antibody coupled to protein A Sepharose. Phosphorylated proteins were separated on an SDS gel and visualized by autoradiography.

**Phosphorylation Site Identification by Nano Liquid Chromatography Tandem Mass Spectrometry**
In vivo phosphorylation of GsaQ was performed as described above in the absence of γ-[32P]ATP. After purification by affinity chromatography, SDS-PAGE, and Coomassie blue staining, bands corresponding to GsaQ were excised from the gel. The gel slices were reduced with dithiothreitol and alkylated with iodoacetamide. Following in-gel trypsin digestion, extracted tryptic peptides were first loaded onto a 300 µm x 5 mm PepMap C18 trap column and backflushed onto a LC-Packings 75 µm x 15 cm C18 nano column at a flow rate of 200 nl/min (LC Packings, Sunnyvale, CA). Peptides were eluted with a 25 min gradient from 10%–40% acetonitrile in 0.1% formic acid followed by 10 min wash at 90% acetonitrile in 0.1% formic acid. The LC was coupled to a QSTAR XL mass spectrometer (Applied Biosystems, Framingham, MA). Mass ranges for the MS survey scan were m/z 500–1200. Mass ranges for MS/MS were m/z 100–1500. The scan times for MS and MS/MS were 1 s and 4 s, respectively. The top multiply charged ions with MS peak intensities greater than 50 counts were chosen for MS/MS fragmentation. MS/MS data was analyzed with Mascot (Matrix Science Inc., Boston, MA), and results were manually verified.

**Membrane Preparation**
Cell membranes were prepared as described by Dowling et al. (2004). Confluent monolayers of transfected HEK293A cells were briefly washed with lifting buffer (10 mM HEPES, 0.9% NaCl, 0.2% EDTA [pH 7.4]) and cells lifted from the tissue culture dish by addition of lifting buffer for approximately 15 min. A cell pellet was recovered by centrifugation at 250 x g for 5 min at 4°C. The cell pellet was homogenized on ice in buffer A (10 mM HEPES, 10 mM EDTA [pH 7.4]) using a Polytron homogenizer (≤20,000 rpm, 5 x 10 s bursts). The homogenate was then centrifuged (5000 x g, 10 min, 4°C), and the resulting supernatant fraction was further centrifuged at 50,000 x g for 15 min at 4°C. The pellet was rehomogenized and recentrifuged as described above. in buffer B (10 mM HEPES, 0.1 mM EDTA [pH 7.4]). The final membrane pellet was resuspended in buffer B, rapidly frozen in liquid nitrogen, and stored at –80°C until needed.

**[35S]GTPyS Binding Assay**
[35S]GTPyS binding assay and subsequent immunoprecipitation were performed according to the method described previously by Dowling et al. (2004). Aliquots of frozen cell membranes were diluted in assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl2 [pH 7.4]) in a total volume of 50 µl. Membranes were added to 50 µl of assay buffer containing 10 nM [35S]GTPyS/S (1250 Ci/mmol), 0.1 mM GDP, and 200 µM carbachol and incubated at 30°C for 30 min. Incubations were terminated by the addition of 1 ml of ice-cold assay buffer and immediately centrifuged (20,000 x g, 6 min, 4°C). Membrane pellets were solubilized by the addition of 50 µl of ice-cold solubilization buffer (100 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1.25% Nonidet P40 [pH 7.4]) containing 0.2% SDS and vortex mixing. Following solubilization, an equal volume of solubilization buffer without SDS was added to each tube.

The solubilized protein was precleared with normal rabbit serum (1:100 dilution) and 30 µl of 1.5% protein A Sepharose suspension for 60 min at 4°C. The protein A beads and any insoluble material were collected by centrifugation at 20,000 x g for 3 min, then 100 µl of the supernatant was immunoprecipitated with an anti-GsaQ antibody (1:100 dilution). Samples were vortex mixed and rotated for 90 min at 4°C. Protein A Sepharose beads (70 µl) were added to each sample and again vortex-mixed and rotated for 90 min at 4°C. Protein A Sepharose beads were then pelleted at 20,000 x g and the supernatant removed by aspiration. The beads were washed three times with 500 µl solubilization buffer without SDS, and after the final wash the recovered beads were mixed with scintillation cocktail and counted. Nonspecific binding was determined in the presence of 10 mM GTPyS.

**Bacterial Infections**
The Y. pseudotuberculosis strains used in this study include YPIII/pB29MEKA(MYM), YPIII/pB29MEKA_C10, pYpkA (MYM + WT YpkA), and YPIII/pB29MEKA_C10, pYpkA(pIE270A) (MYM + YpkA(pIE270A) and have been previously described (Dukuzumuremyi et al., 2000). In our overexpression studies, we used YopO from Y. enterocolitica (called YpkA in Y. pseudotuberculosis); however, the bacterial infections were performed with a Y. pseudotuberculosis strain expressing YpkA.
Y. pseudotuberculosis YpkA kinase mutant (YpkA<sup>D267A</sup>) corresponds to the Y. enterocolitica YopO<sup>D267A</sup> mutant. The reason is that YopO has three extra amino acids in the N terminus, compared to YpkA. We have chosen to use the name “YpkA” instead of “YopO,” since this is the name most commonly used in the literature. HEK293A cells grown on coverslips were transfected 24 hr prior to infection. The Y. pseudotuberculosis strains were grown at 26°C in Luria-Bertani (LB) medium without antibiotics overnight, and 20 μl of the overnight culture was added to 2.5 ml of DMEM supplemented with 10% fetal calf serum and without antibiotics. The inoculated bacterial cultures were grown at 26°C for 30 min followed by incubation for 1 hr at 37°C prior to infection. After 2 hr of infection (multiplicity of infection of approximately 20), the cells were processed for immunofluorescence staining. In experiments using carbachol, the drug was added during the last 30 min of infection.

**Supplemental Data**

Supplemental Data include supplemental text and three figures and can be found with this article online at [http://www.molecule.org/cgi/content/full/26/4/465/DC1/](http://www.molecule.org/cgi/content/full/26/4/465/DC1/).

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