Regulation of *Yersinia* Protein Kinase A (YpkA) Kinase Activity by Multisite Autophosphorylation and Identification of an N-terminal Substrate-binding Domain in YpkA*

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**Background:** The catalytic mechanism of the *Yersinia* protein kinase YpkA is poorly understood. **Results:** Multiple N-terminal autophosphorylation sites regulate YpkA activation and residues 40–49 of YpkA contribute to Goq binding and phosphorylation. **Conclusion:** The N-terminal domain of YpkA plays a role in autophosphorylation and substrate binding. **Significance:** Elucidating how type III bacterial effectors are regulated is essential to our understanding of infectious diseases.

The serine/threonine protein kinase YpkA is an essential virulence factor produced by pathogenic *Yersinia* species. YpkA is delivered into host mammalian cells via a type III secretion system and localizes to the inner side of the plasma membrane. We have previously shown that YpkA binds to and phosphorylates the α subunit of the heterotrimeric G protein complex, Goq, resulting in inhibition of Goq signaling. To identify residues in YpkA involved in substrate binding activity we generated GFP-YpkA N-terminal deletion mutants and performed coimmunoprecipitation experiments. We located a substrate-binding domain on amino acids 40–49 of YpkA, which lies within the previously identified membrane localization domain on YpkA. Deletion of amino acids 40–49 on YpkA interfered with substrate binding, substrate phosphorylation and substrate inhibition. Autophosphorylation regulates the kinase activity of YpkA. To dissect the mechanism by which YpkA transmits signals, we performed nano liquid chromatography coupled to tandem mass spectrometry to map *in vivo* phosphorylation sites. Multiple serine phosphorylation sites were identified in the secretion/translocation region, kinase domain, and C-terminal region of YpkA. Using site-directed mutagenesis we generated multiple YpkA constructs harboring specific serine to alanine point mutations. Our results demonstrate that multiple autophosphorylation sites within the N terminus regulate YpkA kinase activation, whereas mutation of serine to alanine within the C terminus of YpkA had no effect on kinase activity. YpkA autophosphorylation on multiple sites may be a strategy used by pathogenic *Yersinia* to prevent inactivation of this important virulence protein by host proteins.

Three *Yersinia* species (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) are highly pathogenic for humans. All three harbor an extrachromosomal 70 kb plasmid that encodes a type III secretion system (T3SS) and a variety of bacterial virulence factors. The T3SS is a sophisticated translocation apparatus highly conserved among many Gram-negative bacteria that is used to deliver bacterially encoded proteins directly into the host cytosol (1). *Yersinia* uses the T3SS to deliver a set of effector proteins termed Yops (*Yersinia* outer proteins) into infected eukaryotic cells: YopH, a protein tyrosine phosphatase; YpA (referred to as YopO in *Y. enterocolitica*), a protein kinase; YopT, a cysteine protease; YopJ (referred to as YopP in *Y. enterocolitica*), an acetyltransferase; YopE, a GTPase-activating protein; and YopM, a leucine-rich protein that down-regulates expression of pro-inflammatory cytokines (2–12). Once inside the host cell, the concerted activities of the Yop effectors function to prevent phagocytosis, superoxide production, and cytokine synthesis by professional phagocytes and other types of host cells by directly interacting with host proteins and inhibiting signaling pathways (13–19).

YpkA is a serine/threonine protein kinase that phosphorylates actin and the small heterotrimeric G protein subunit Goq (20–21). The C terminus of YpkA interacts with members of the Rho family of small GTPases, RhoA and Rac1 (22–23). The deubiquitinating enzyme otubain 1 (OTUB1) was initially identified as a substrate for YpkA; however more recent studies have linked otubain 1 to the RhoGDI domain of YpkA (24–25). The 729-amino acid YpkA protein is composed of multiple domains (Fig. 1). Residues 1–77 (Sec/Trans) mediate type III secretion and translocation of YpkA into a target cell (26). This region coincides with a chaperone-binding domain (CBD), amino acids 20–77 (27). Once inside the cell, a membrane localization domain (MLD) consisting of amino acids 20–90 localizes YpkA to the inner side of the plasma membrane where it is in close proximity to signaling proteins involved in transducing external signals inside the cell (27–28). Residues 150–400 comprise the N-terminal serine/threonine kinase domain. Substitution

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**Note added in proof:** Although we initially identified a substrate for YpkA, further studies have linked this substrate to the RhoGDI domain of YpkA. YpkA is a serine/threonine protein kinase that phosphorylates actin and the small heterotrimeric G protein subunit Goq (20–21). The C terminus of YpkA interacts with members of the Rho family of small GTPases, RhoA and Rac1 (22–23). The deubiquitinating enzyme otubain 1 (OTUB1) was initially identified as a substrate for YpkA; however more recent studies have linked otubain 1 to the RhoGDI domain of YpkA (24–25). The 729-amino acid YpkA protein is composed of multiple domains (Fig. 1). Residues 1–77 (Sec/Trans) mediate type III secretion and translocation of YpkA into a target cell (26). This region coincides with a chaperone-binding domain (CBD), amino acids 20–77 (27). Once inside the cell, a membrane localization domain (MLD) consisting of amino acids 20–90 localizes YpkA to the inner side of the plasma membrane where it is in close proximity to signaling proteins involved in transducing external signals inside the cell (27–28). Residues 150–400 comprise the N-terminal serine/threonine kinase domain. Substitution

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2 The abbreviations used are: T3SS, type III secretion system; YpkA, *Yersinia* protein kinase A; OTUB1, otubain 1; CBD, chaperone-binding domain; MLD, membrane localization domain; GDI, guanine nucleotide dissociation inhibitor; GPCR, G protein-coupled receptor.
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of a conserved aspartic acid (Asp-267) and a lysine (Lys-269) residue with alanine results in a catalytically inactive kinase (21). Y. pseudotuberculosis mutant strains expressing catalytically inactive YpkA variants are markedly attenuated in virulence in mouse infection studies (19). In cell culture infection assays, the enzymatic activity of YpkA was necessary for inhibition of host cell bacterial internalization (29–31). A region within the C-terminal domain (residues 431–612, RhoGDI) of YpkA possesses Rho GTPase binding guanine nucleotide dissociation inhibitor (GDI)-like activity and has been shown to be important for inactivation of the small Rho GTPases, RhoA and Rac1 (32). The GDI-like activity interferes with phagocytosis by disrupting the host actin cytoskeleton (33). Substitution of three amino acids (Y591A, N595A, E599A) in the GDI-like domain interferes with Rho GTPase binding (32). The last 21 amino acids (residues 709–729) are involved in actin binding and subsequent autoactivation of YpkA kinase activity (21). Residues serine 90 and serine 95 were reported as autophosphorylation sites required for efficient activation and phosphorylation of exogenous substrates by YpkA (30).

Both kinase and guanine nucleotide dissociation inhibitor domains of YpkA are important in the activity of full length YpkA (19, 31–32). The kinase activity of YpkA is dependent on its association with actin (21, 30). Although YpkA has been shown to phosphorylate actin and otubain 1 in vitro, the physiological importance of these findings is unclear (21, 24, 25). We previously reported that YpkA interacts with and phosphorylates the heterotrimeric G protein Goq, although the involvement of additional components remains to be determined (20). YpkA-mediated phosphorylation of Ser-47 on Goq impairs guanine nucleotide binding and subsequently inhibits Goq-mediated signaling pathways (20). Goq belongs to the family of heterotrimeric G proteins that 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 (34–35). G proteins are divided into four families based on sequence similarities of the α subunits: Gas, Gai/o, Goα12/13, and Goq. Members of the Gas and Gai families are known to activate and inhibit adenyl cyclase, respectively. Members of the Goα12/13 family regulate the small G protein RhoA, while Goq family members stimulate phospholipase C-β (PLC-β), leading to the hydrolysis of phosphatidyl-4,5-bisphosphate and the production of inositol triphosphate (IP3) and diacylglycerol (DAG). In addition, Goq family members have also been shown to activate RhoA-mediated pathways. The importance of heterotrimeric G protein α subunits in eukaryotic defense responses is underscored by the observation that a number of bacterial pathogens have evolved toxins that specifically target their activity (36).

We have previously shown that the N-terminal 430 amino acids of YpkA are essential for substrate binding (20). As a first step toward elucidating the mechanism of substrate recognition mediated by the YpkA N-terminal domain, we have identified residues 40–49 that are critical for YpkA-mediated inhibition of Goq signaling. The efficiency of substrate-binding and -phosphorylation by YpkA is diminished by deletion of residues 40–49 of YpkA, suggesting that they are important for substrate recognition. Trasak et al. proposed a model in which actin binding induces autophosphorylation of YpkA on serine 90 and serine 95 (30). Using an in vivo labeling assay we demonstrated that a YpkA S90A/S95A mutant undergoes autophosphorylation and demonstrates substrate phosphorylation activity, indicating the presence of additional autophosphorylation sites. Here, we report that multiple autophosphorylation sites within the N terminus of YpkA regulate its kinase activity. These findings further our understanding of the molecular mechanism used by Yersinia type III effectors to circumvent host defenses.

Experimental Procedures

Cell Culture, Transfection, and Reagents—Human embryonic kidney cells (HEK293A) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, and 2 mM l-glutamine. Cells were cultured in a humidified atmosphere of 5% CO2 at 37 °C. The TransIT-LT1 Transfection Reagent (Mirus) or the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) was used according to the manufacturer’s recommendations. All reagents were from Fisher Scientific, Sigma-Aldrich, Invitrogen, or New England Biolabs unless otherwise noted. All oligonucleotide primers were from Integrated DNA Technologies.

Construction of 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), FLAG-tagged pcDNA3.1 (Invitrogen), or GST-tagged pGEX-6P-2 vectors following standard protocols. YpkA internal deletion mutants were generated using the In-Fusion HD Cloning Kit (Clontech) following the manufacturer’s instructions. All point mutations were introduced by using the QuikChange II Site-directed Mutagenesis Kit (Agilent) following the manufacturer’s recommendations. All expression constructs were verified by sequencing.

Recombinant Protein Expression—Expression of YpkA with GST was induced in subcultures of Escherichia coli BL21 (RIPL) using 0.4 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG). After overnight incubation at room temperature, bacteria were pelleted and resuspended in GST lysis buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, and 2 mM dithiothreitol), 1 mM phenylmethylsulfonyl fluoride, and Halt Protease inhibitor mixture (Thermo Scientific). Bacterial cells were lysed using an Avestin EmulsiFlex-C3 homogenizer. The crude lysate was...
incubated with 0.2% Triton X-100 prior to pelleting the bacterial debris. The GST-tagged protein was affinity-purified on glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer’s instructions. Bead-bound proteins were pelleted, washed with GST lysis buffer, and eluted with 200 mM reduced glutathione (in GST lysis buffer, pH 7.4).

**Immunoprecipitation and Immunoblotting**—After transfection for 16–20 h, cells were lysed in modified RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM sodium fluoride, and 0.4 mM EDTA) containing protease inhibitors (Halt Protease Inhibitor Mixture; Thermo Scientific) and 1 mM PMSF. The homogenate was centrifuged (14,000 x g, 4°C, 20 min), and the supernatants were incubated with anti-FLAG M2 agarose beads (Sigma) or with the indicated antibodies bound to protein G beads overnight at 4°C with gentle rotation. After incubation, immunoprecipitates were washed extensively with ice-cold modified RIPA buffer. Proteins bound to the beads were eluted by heating at 70°C for 10 min in LDS-PAGE sample loading buffer. The eluted proteins were separated by SDS/PAGE, transferred to a PVDF membrane, and probed with the specified antibodies followed by chemiluminescence detection. Whole cell lysates were separated by SDS/PAGE and subjected to immunoblotting as described above. The following antibodies were used: anti-actin (20–33) (Sigma), anti-GFP (JL-8) (Clontech), anti-FLAG-M2 (Sigma), and anti-Goq (E-17) (Santa Cruz Biotechnology).

**Generation of Goq Phosphospecific Antibodies**—Polyclonal affinity purified phosphospecific peptide antibodies were generated by 21st Century Biochemicals (Marlboro, MA). The Goq peptides used were: TGESGK[pS]TFIKQMC and CGTGESGK[pS]TFIKQM. To selectively purify antibodies with phosphospecificity from the anti-sera, a two-stage affinity purification was taken. First, each anti-serum was negatively purified by exposure to an affinity column containing the nonphosphorylated peptide. Second, the flow-through from the negative purification was positively purified on a column containing the phosphorylated peptide.

**Immunofluorescence**—Semiconfluent HEK293A monolayers were grown overnight on 22-mm-diameter glass cover slips in DMEM supplemented with 10% fetal bovine serum. Monolayers were transfected with plasmid DNA as described above. For all carbachol (Calbiochem) experiments, HEK293A cells were also transfected with HA-M1 muscarinic receptor (M1R) cDNA expression plasmid to make these cells responsive to carbachol since they do not express endogenous M1Rs. Where indicated cells were stimulated with 200 μM carbachol for 60 min. For indirect immunofluorescence, samples were fixed, permeabilized, and stained as previously described (21). Proteins were visualized by direct fluorescence of GFP- or mCherry-containing proteins, or, where indicated, with anti-FLAG M2-Cy3 (Sigma), and the appropriate secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). Nuclear staining was achieved by staining with Hoescht stain (Invitrogen). Rhodamine-phalloidin (Invitrogen) was used to stain the actin cytoskeleton. Images were acquired by epi-fluorescence microscopy with the ×60 apochromat objective lens using a Nikon Eclipse 80i fluorescence microscope. For subcellular localization of the YpkA variants an Olympus FV1000 laser scanning confocal microscope was used to image the cells.

**In Vivo Labeling with [32P]Orthophosphate**—Labeling experiments were performed as described previously (20). For all labeling experiments 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-h incubation with the same medium containing [32P]Orthophosphate (150 μCi/ml), the cells were lysed with TNN lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA) containing Halt Protease Inhibitor Mixture (Thermo Scientific) and 1 mM PMSF. Whole-cell lysates were harvested and split equally. One-half was incubated with anti-FLAG agarose beads. The remaining lysate was used in an immunoprecipitation with a polyclonal antiserum bound to protein A-Sepharose that recognizes Goq. Bead-bound proteins were washed with lysis buffer, heated in LDS sample buffer at 70°C for 10 min, and separated by SDS-PAGE. Protein phosphorylation was visualized by autoradiography. For plasmid expression, an immunoblot was performed as described above on whole-cell lysates. Densitometry was performed using the ImageJ analysis software (NIH) as per the developer’s recommendations.

**Phosphorylation Site Identification by Nano Liquid Chromatography Tandem Mass Spectrometry**—In vivo phosphorylation of FLAG-YpkA 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 YpkA were excised from the gel. YpkA was prepared for MS analysis using methods adapted from standard reduction, alkylation, and tryptic digestion procedures (37). Peptides were dried down in a vacuum concentrator after digestion, then resolubilized in 2% acetonitrile/0.1% trifluoroacetic acid for LC-MS/MS analysis. Digested peptides were analyzed by LC-MS/MS on an LTQ-FT with Michrom Paradigm LC and CTC Pal autosampler. Peptides were directly loaded onto a Agilent ZORBAX 300SB C18 reversed phase trap cartridge, which, after loading, was switched in-line with a Michrom C18 column connected to the Thermo-Finnigan LTQ-FT mass spectrometer through a Michrom Advance Plug and Play nano-spray source. The nano-LC column (Michrom 3μ 200Å MAGIC C18AQ 200μ × 150 mm) was used with a 90-min gradient (2–10% buffer B in 5 min, 10–35% buffer B in 65 min, 35–70% buffer B in 5 min, hold at 70% buffer B for 1 min, then down to 2% buffer B in 1 min, holding at 2% buffer B for 13 min) at a flow rate of 2 μl min⁻¹ for the maximum separation of tryptic peptides. MS and MS/MS spectra were acquired using a top 4 method and an MS survey scan was obtained for the m/z range 400–1300. An isolation mass window of 2 Da was for the precursor ion selection, and a normalized collision energy of 35% was used for the fragmentation. Tandem mass spectra were extracted by Xcalibur version 2.0.7. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version CYCLONE (2013.02.01.1)). X! Tandem was set up to search a Yersinia database (9010 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.40 Da and a
parent ion tolerance of 20 PPM. Carbamidomethyl of cysteine
was specified in X!Tandem as a fixed modification. Glu-
pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, deami-
dation of asparagine and glutamine, oxidation of methionine
and tryptophan, acetyl of the N-terminus and phosphorylation
of serine, threonine, and tyrosine were specified in X!Tandem
as variable modifications. Scaffold (version Scaffold_4.0.1,
Proteome Software Inc., Portland, OR) was used to validate
MS/MS based peptide and protein identifications. Peptide
identifications were accepted if they met or exceeded 95% prob-
ability in Scaffold. Protein identifications were accepted if they
were greater than or equal to 95% probability determined in
Scaffold, and they contained at least 2 identified peptides.
Proteins that contained similar peptides and could not be dif-
ferentiated based on MS/MS analysis alone were grouped to
satisfy the principles of parsimony. Proteins sharing significant
peptide evidence were grouped into clusters. Two LC-MS/MS
runs on the LTQ-FT were used to create the list of possible
phosphorylation sites.

RESULTS

Identification of a Novel Substrate-binding Domain at the N
Terminus of YpkA—We previously demonstrated that the
YpkA N-terminal residues 1–430 mediate interaction with Gq (20).
To further define the substrate-binding domain we
generated GFP-tagged N-terminal YpkA truncations and per-
formed immunoprecipitation assays. As previously shown both
full-length YpkA and a YpkA variant containing the kinase
domain (amino acids 1–430) interacted with Gq, whereas a
C-terminal YpkA variant containing the GDI-like domain
(amino acids 399–729) was unable to interact with Gq (Fig.
2A). Furthermore, whereas the N-terminal deletion mutant
YpkA40–729 associated with Gq (Fig. 2A, lane 5), further dele-
tion at the N terminus of YpkA resulted in loss of binding to

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FIGURE 2. The N terminus of YpkA reveals a novel substrate-binding site. A, residues 40–88 of YpkA harbor a substrate-binding site. HEK293A cells were
cotransfected with GFP-YpkA constructs and Gq. Anti-GFP immunoprecipitations (IP) were probed by anti-Gq or GFP immunoblot (IB) to show input
levels. B, amino acids 40–49 of YpkA are essential for substrate binding. The GFP-tagged YpkA deletion constructs were coexpressed with Gq in HEK293A
cells. The binding of truncated versions of YpkA to Gq, and the immunoprecipitated YpkA mutants, as well as the level of protein expression in whole cell
lysates, were analyzed by Western blotting with anti-GFP and anti-Gq antibodies. C, residues 1–150 of YpkA are not sufficient for Gq binding. HEK293A cells
overexpressing Gq alone or with one of the indicated GFP-tagged constructs were lysed, and immunoprecipitation was performed using the anti-GFP
antibody. Immunoblots were carried out using anti-GFP and anti-Gq antibodies. (* denotes IgG heavy chain; Gq, 42 kDa; YpkA, 108.3 kDa; YpkA, 74.4
kDa; YpkA, 64.4 kDa; YpkA, 104 kDa; YpkA, 102.6 kDa; YpkA, 101.5 kDa; YpkA, 99.3 kDa; YpkA, 98.4 kDa; YpkA, 97 kDa; YpkA, 92 kDa; YpkA, 91 kDa;
YpkA, 84.4 kDa; YpkA, 71 kDa; YpkA, 64.4 kDa; YpkA, 53 kDa; YpkA, 44 kDa).
Thus, amino acids 40–88 of YpkA are required for efficient binding of Gq, since deletion of the first 88 amino acids prevented Gq binding. To further define the region responsible for substrate binding we created additional N-terminal YpkA deletion mutants (Fig. 2B). As compared with the efficient substrate binding by YpkA_{40–729} (Fig. 2B, lane 5), removal of an additional ten amino acids (YpkA_{50–729}) was sufficient to disrupt Gq binding (Fig. 2B, lanes 6–10). Controls in this experiment included full-length YpkA and YpkA_{1–430} that bound to Gq (Fig. 2B, lanes 2–3), as opposed to YpkA_{399–729} that did not (Fig. 2B, lane 4). N-terminal YpkA peptides fused to GFP did not associate with Gq suggesting that additional residues play a role in substrate binding (Fig. 2C, lanes 5–7).

Amino acids 40–50 of YpkA Are Required for Efficient Inhibition of Gq Signaling—YpkA binds and phosphorylates the heterotrimeric G protein Gq interfering with GTP binding and activation (20). We examined the effect of the YpkA N-terminal deletion mutants on Gq signaling using a tubby nuclear translocation assay to monitor activation of Gq (20). The transcription factor tubby is involved in maturity-onset obesity in mice and is known to be a downstream target of Gq (38). Tubby localizes to the plasma membrane by binding phosphatidylinositol 4,5-bisphosphate. Receptor-mediated activation of Gq is thought to release tubby from the plasma membrane through the activity of phospholipase C-β, triggering translocation of tubby to the nucleus (38). HEK293A cells were transfected with a mCherry-tagged tubby plasmid and either GFP, GFP-YpkA, GFP-YpkA_{D267A} or a GFP-YpkA N-terminal deletion mutant. After overnight transfection, HEK293A cells were stimulated with carbachol for 60 min. Then cells were washed, fixed, permeabilized, and visualized via immunofluorescence. Expression of GFP in control cells did not interfere with Gq-mediated nuclear localization of tubby (Fig. 3A, panel A). As previously shown, expression of YpkA interfered with the nuclear translocation of tubby after carbachol stimulation (Fig. 3A, panel B), whereas the catalytically inactive YpkA mutant, YpkA_{D267A}, did not prevent localization of tubby to the nucleus confirming that the kinase activity of YpkA is required for inhibition of Gq signaling (Fig. 3A, panel C). Although YpkA_{1–430}
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binds Gao, it does not interfere with its activation (Fig. 3A, panel D). The GDI-like domain was not sufficient to prevent the nuclear localization of tubby (Fig. 3A, panel E). Importantly, YpkA20–729 interacts with Gao and inhibits its activation (Fig. 3A, panel F). However, YpkA50–729 did not inhibit Gao mediated nuclear translocation of tubby, nor did further N-terminal deletion mutants (Fig. 3A, panels G–M). Therefore, residues 40–50 of YpkA appear critical for inhibition of Gao.

Since autophosphorylation is a requirement for YpkA kinase activation and subsequent inhibition of Gao signaling, we confirmed the ability of these N-terminal YpkA mutants to auto-phosphorylate in an in vitro kinase assay (Fig. 3B) (30). Bacterially expressed GST-YpkA proteins were generated and subjected to an in vitro kinase assay. Autophosphorylation was observed in wild-type YpkA and YpkA100–729. In contrast, autophosphorylation was not observed with either the kinase inactive mutant, YpkA1267A, or the N-terminal deletion mutant YpkA151–729 suggesting that amino acids 100–150 are required for autophosphorylation, since deletion of amino acids 1–99 did not affect YpkA kinase activity. Thus, the inability of YpkA50–729 to interfere with Gao signaling is likely due to reduced substrate binding rather than improper folding of the protein (Fig. 3B).

We next examined the effect of deleting amino acids 40–50 of YpkA on Gao binding and phosphorylation. Immunoprecipitates prepared from HEK293A cells transfected with vector, FLAG-YpkA, FLAG-YpkA40–50 or FLAG-YpkA1–430 were analyzed by SDS/PAGE and immunoblotting. YpkA and YpkA1–430 associated with Gao, whereas YpkA40–50 had minimal binding to Gao (Fig. 4A). To detect phosphorylation of Gao by YpkA we used an antibody generated against phospho-Gao (Ser-47). Phosphorylated Gao was detected in lysates of YpkA-transfected HEK293A cells. However, phosphorylation of Gao was significantly reduced in cell lysates expressing YpkA40–50 (Fig. 4B). We further tested the effect of YpkA40–50 on the nuclear translocation of tubby upon carbachol stimulation (Fig. 4C). Tubby nuclear translocation was seen in control cells as well as in cells expressing the catalytically deficient mutant, YpkA1267A. Wild-type YpkA interfered with tubby nuclear localization, whereas, YpkA40–50 lost ability to inhibit Gao mediated translocation of tubby to the nucleus, suggesting that amino acids 40–50 on YpkA enhance the efficiency of substrate phosphorylation.

The Substrate-binding Domain Resides within the Membrane Localization Domain of YpkA—Upon translocation into a target cell by the Yersinia T3SS, YpkA localizes to the inner side of the plasma membrane (28). Previous studies reported that the MLD of YpkA resides within amino acids 20–90. Since the substrate-binding domain lies within this region we examined the effect of a YpkA20–90 internal deletion on Gao binding, phosphorylation and signaling. As shown in Fig. 5A, YpkA20–90 was deficient in Gao binding and phosphorylation. Additionally, YpkA20–90 did not interfere with Gao-mediated nuclear translocation of tubby (Fig. 4C). To determine if deletion of amino acids 40 through 50 interfered with membrane localization we transfected HEK293A cells with vector, FLAG-YpkA, FLAG-YpkA1–430, FLAG YpkA40–50 and FLAG-YpkA20–90 and performed immunofluorescence microscopy. After overnight transfection, cells were fixed, washed and permeabilized. YpkA was detected by FLAG-Cy3 antibody. YpkA was detected by an antibody generated against phospho-Gao (Ser-47). Phosphorylated Gao by YpkA we used an antibody generated against phospho-Gao (Ser-47). Phosphorylated Gao was detected in lysates of YpkA-transfected HEK293A cells. However, phosphorylation of Gao was significantly reduced in cell lysates expressing YpkA40–50 (Fig. 4B). We further tested the effect of YpkA40–50 on the nuclear translocation of tubby upon carbachol stimulation (Fig. 4C). Tubby nuclear translocation was seen in control cells as well as in cells expressing the catalytically deficient mutant, YpkA1267A. Wild-type YpkA interfered with tubby nuclear localization, whereas, YpkA40–50 lost ability to inhibit Gao mediated translocation of tubby to the nucleus, suggesting that amino acids 40–50 on YpkA enhance the efficiency of substrate phosphorylation.
YpkA S90A/S95A Autophosphorylation Mutant Possesses Kinase Activity in Vivo—YpkA is found in a catalytically inactive conformation when produced in *Yersinia* (21). Full activation of YpkA kinase activity in vitro requires actin binding and autophosphorylation. Trasak et al. proposed a model in which actin binding induces autophosphorylation of YpkA on serine 90 and serine 95 (30). A YpkAS90A/S95A mutant was reduced in autophosphorylation and phosphorylation of exogenous substrates in an in vitro kinase assay (30). To assess whether autophosphorylation on serine 90 and serine 95 were required for phosphorylation of Gq/H9251q we examined the affect of a YpkAS90A/S95A mutant on Gq/H9251q activation. HEK293A cells were transfected with GFP-tubby and either vector, YpkA, YpkAD267A, or YpkAS90A/S95A and a tubby nuclear translocation assay was performed. After overnight transfection, the cells were stimulated with carbachol for 60 min and processed for immunofluorescence microscopy. Tubby nuclear localization was evident in cells expressing vector and YpkAD267A only (Fig. 6A). Wild-type YpkA and YpkAD267A inhibited the nuclear translocation of tubby after carbachol stimulation (Fig. 6A).

To confirm that YpkAS90A/S95A underwent autophosphorylation we performed metabolic labeling experiments to assess YpkA autophosphorylation and Gq phosphorylation by YpkA in vivo. HEK293A cells were transiently transfected with Gq/H9251qQ209L in the presence of either vector, wild-type YpkA, YpkAD267A or YpkAS90A/S95A. Following a 2-h incubation with [32P]orthophosphate, YpkA and Gq were immunoprecipitated from cell lysates with anti-FLAG and anti-Gq antibodies, respectively. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. As shown in Fig. 6B, incorporation of [32P]orthophosphate into the YpkA protein was observed in wild-type YpkA and YpkAS90A/S95A, but not in vector controls or upon exposure of equal amounts of the inactive kinase-deficient YpkAD267A. Additionally, Gq was phosphorylated by all YpkA variants except in vector controls and YpkAD267A lysates. Thus, autophosphorylation on serine 90 and serine 95 on YpkA are not critical for Gq/H9251q phosphorylation. Taken together, these results allude to the presence of additional autophosphorylation sites on YpkA and underscore the complexity of this *Yersinia* effector.
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agarose beads. Following gel electrophoresis, immunoprecipitated FLAG-YpkA was extracted from the gel followed by nano liquid chromatography tandem mass spectrometry. We identified nineteen autophosphorylation sites, two of which were previously reported (Fig. 7A) (30). These residues were located in the secretion/translocation region, kinase domain, and RhoGDI domain of YpkA. We initially generated a full-length YpkA mutant containing serine to alanine point mutations in all nineteen residues, YpkASA (Fig. 7A). FLAG-YpkA, FLAG-YpkA$_{1267A}$ and FLAG-YpkA$_{SA}$ were immunoprecipitated from transiently transfected 293A human embryonic kidney cells and autophosphorylated in vitro in a kinase buffer containing [γ-$^{32}$P]ATP. Wild-type YpkA underwent autophosphorylation, whereas, YpkA$_{1267A}$ and YpkA$_{SA}$ were deficient in their ability to incorporate radioactivity (Fig. 7B). We next determined whether YpkA$_{SA}$ was affected in its ability to phosphorylate Gq by performing a Western blot using a phospho-Gq antibody. Gq phosphorylation was significantly reduced in HEK293A lysates expressing YpkA$_{SA}$ compared with lysates expressing YpkA (Fig. 7C, second panel). Lack of Gq phosphorylation was not due to its inability to bind to Gq as shown in Fig. 7C (first panel). These results imply that YpkA$_{SA}$ may harbor critical residues required for YpkA autophosphorylation. We next generated four additional YpkA serine to alanine mutants: YpkASA$_{1–150}$ contains S52A, S55A, S90A, S95A, S102A, S144A, and S147A; YpkASA$_{150–400}$ contains S2A, S55A, S90A, S95A, S102A, S144A, S147A, S164A, S303A, S317A, S320A, S327A, S353A, and S389A; YpkASA$_{436–710}$ contains S496A, S520A, S529A, S534A, and S620A. FLAG-tagged proteins were expressed in HEK293A cells and immunoprecipitated with anti-FLAG. Immunoprecipitates were subjected to an in vitro kinase assay and followed by SDS-PAGE. The level of autophosphorylation was similar for YpkA and YpkASA$_{436–710}$ suggesting that the indicated point mutations within the RhoGDI domain did not affect YpkA autophosphorylation (Fig. 7D). As we previously observed, YpkA$_{SA}$ had minimal levels of autophosphorylation. The YpkA$_{SA–400}$ mutant spanning the secretion/translocation and kinase domains was significantly impaired in its autophosphorylation activity. The levels of autophosphorylation in the YpkA$_{SA1–150}$ and YpkA$_{SA150–400}$ mutants were .52 and .26, respectively, relative to wild type YpkA. We examined the effect of the YpkA variants on Gq signaling in a cellular environment by assessing their ability to interfere with the nuclear translocation of GFP-tubby upon carbachol stimulation. Empty vector control cells displayed nuclear localization of tubby, whereas cells expressing YpkASA$_{1–400}$ to inhibit the nuclear translocation of tubby shows a direct correlation with the level of autophosphorylation activity. In the presence of YpkA$_{SA150–400}$ and YpkA$_{SA436–710}$ tubby did not accumulate in the nucleus. Altogether, our results suggest that multisite autophosphorylation is an important determinant in the regulation of YpkA kinase activity.

DISCUSSION

Identification of a Novel Substrate-binding Site at the N Terminus of YpkA—Virulence in Y. pseudotuberculosis depends upon the translocated virulence factor YpkA, a protein that disrupts the actin cytoskeleton and inhibits phagocytosis (13).
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Despite the known importance to virulence, little information has been forthcoming to elucidate the mechanism of activity of YpkA. This has been particularly true of the N-terminal domain of the protein, which, while known to have serine/threonine kinase activity, has remained enigmatic in terms of function. In addition to a kinase domain, the N-terminal domain of YpkA also contains a membrane localization domain, amino acids 20–90, and a chaperone-binding domain, amino acids 20–77 (27–28). In this study, we have determined by deletion experiments that a region between residues 40–49 of YpkA is critical for efficient substrate-binding and phosphorylation, and inhibition of Gαq signaling. The low levels of Gαq observed in Fig. 2B, lanes 6–10, suggest that, although, amino acids 40–49 on YpkA enhance the efficiency of substrate binding additional residues may also contribute to substrate binding. Our results using YpkA peptides indicate that the substrate-binding domain is likely “discontinuous” and is dependent on the secondary and tertiary structure of YpkA. Thus, the N terminus of YpkA serves multiple functions for this type III effector.

Multifunctional domains of T3SS effector proteins are an emerging theme in Yersinia. For example, the Yersinia T3SS effector YopH is a 468-amino acid protein tyrosine phosphatase, responsible for disruption of focal adhesions and inhibition of integrin-mediated bacterial phagocytosis (9–11). The N-terminal 129 amino acids of YopH comprise chaperone- and substrate binding activities (39). Additionally, the first 100 residues of YopT, a Yersinia cysteine protease that affects the host actin cytoskeleton by targeting Rho GTPases, contain a chaperone binding site and are essential for binding to Rho GTPases (40). Thus, Yersinia has evolved bacterial virulence factors to exploit the multifunctionality of a single modular domain, as is the case for the YpkA, YopH, and YopT N-terminal domains.

Membrane Localization and Substrate Specificity—Yersinia effector proteins are translocated to different subcellular locations within the target cell, emphasizing the complexity of the strategy used by Yersinia to neutralize host defenses. YopE, a Yersinia GTPase-activating protein for RhoA, Rac1 and Cdc42, is targeted to the perinuclear region of a cell, where all three GTPases are localized (28). YopH localizes to focal adhesion complexes, where it is in close proximity to its substrates (28). Yersinia-delivered YopT is targeted to the plasma membrane, where RhoA is located (7). Upon translocation into the host cell, the N terminus 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. Groves et al. demonstrated the importance of YpkA subcellular localization to the plasma membrane for RhoGTPase binding (33). YpkA was shown to selectively inhibit Rac-dependent Fcγ receptor-mediated phagocytosis by specifically targeting endogenous membrane-bound Rac isoforms in cells (33). More importantly, an overexpressed YopO localization deficient mutant (YopO_{200-77}) had significantly reduced anti-phagocytic ability upon challenge with IgG-sRBC (33). Our findings provide strong evidence for the importance of plasma membrane localization for interaction of YpkA with its cognate host targets. Heterotrimeric G proteins are covalently modified at or near their N termini by covalent attachment of the fatty acids myristate and/or palmitate (41). Palmitoylation on C9 and C10 of Gαq is required for membrane attachment. YpkA interferes with G protein-coupled receptor signaling by inactivating the heterotrimeric G protein Gαq. YpkA binds and phosphorylates Gαq on a critical serine residue preventing GTP binding (20). We have deter-

FIGURE 7. Multiple N-terminal autophosphorylation sites stimulate YpkA kinase activity. A, summary of the mass spectrometry results. FLAG-YpkA was immunoprecipitated from transfected HEK293A cells with anti-FLAG agarose beads. After purification by affinity chromatography, SDS-PAGE, and Coomassie Blue staining, bands corresponding to YpkA were excised from the gel and subjected to nano liquid chromatography tandem mass spectrometry as described in “Experimental Procedures.” Nineteen potential auto-phosphorylation sites were identified, including two previously reported (Ser-90 and Ser-95) (30). The YpkA serine to alanine (YpkASA) mutants were generated using overlap extension PCR. B, autophosphorylation of wild type and mutant YpkA proteins. Immunoprecipitated FLAG-YpkA constructs were subjected to an in vitro kinase assay, run on SDS-PAGE and blotted onto PVDF membrane. Shown are an autoradiograph (upper panel) and an immunoblot probed with an anti-FLAG antibody (lower panel). C, YpkASA is deficient in Gαq phosphorylation. FLAG-tagged YpkA proteins and Gαq were expressed in HEK293A cells, and cell lysates were immunoprecipitated with anti-FLAG. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with antibodies anti-Gαq (first panel) and anti-FLAG (fourth panel). Whole cell lysates were probed with phosopho-Gαq (second panel) and Gαq (third panel) antibodies. *, nonspecific band. D, autophosphorylation of YpkA constructs harboring the indicated serine to alanine point mutations. HEK293A cells were transfected with YpkA or the indicated YpkA variants. Cells were lysed, and anti-FLAG immunoprecipitates were subjected to an in vitro kinase assay. Samples were analyzed by autoradiography (upper panel) and anti-FLAG immunoblotting (lower panel). The numbers indicate levels of autophosphorylation of the YpkA constructs relative to YpkA (control). E, N-terminal serine residues are required for in vivo YpkA activity. Merged image of HEK293A cells transfected with GFP-tubby (green) and vector or indicated YpkA variants. Hoechst staining (blue) was used to detect nuclear staining, and rhodamine phalloidin was used to detect actin (red).
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examined that in addition to Gαq, YpkA associates with other members of the Gαq family, but not with members of the Gα12/13, Gai or Gas families (data not shown). Thus, YpkA exhibits substrate specificity and acts only on a defined subset of cellular targets. Our results demonstrate that substrate binding is not a requirement for appropriate subcellular localization of YpkA. The molecular mechanism used for membrane localization by YpkA is unknown. Recently, Salomon et al. identified a conserved bacterial phosphoinositide-binding domain (BPD) present in type III effectors of both animal and plant pathogens, including YpkA. However, a YpkA truncation mutant containing a mutation of a conserved tyrosine residue (Tyr41 in Y. pseudotuberculosis and Tyr-38 in Y. enterocolitica) within the BPD could not be examined for membrane localization due to lack of expression (42). We speculate that additional YpkA kinase substrates will be targeted to the plasma membrane. Thus, the membrane localization domain mediates the full effect of YpkA function by contributing to both the kinase activity and the GDI activity of the C-terminal domain.

Multisite Phosphorylation Regulates Kinase Activity—Our data indicate that YpkA kinase activity is regulated by multisite autophosphorylation within its N-terminal domain (amino acids 1 to 400). We observed the level of autophosphorylation for the YpkA<sub>SA1-400</sub> and YpkA<sub>12267A</sub> mutants to be similar. Based on our results it seems likely that serine residues within the first 150 amino acids of YpkA (YpkA<sub>SA1-150</sub>) and within amino acids 150 to 400 are necessary for full activation of YpkA kinase activity. Additional one, two and three point mutations within these regions to narrow down the critical sites reduced the level of YpkA autophosphorylation, but had no effect on tubby nuclear translocation (data not shown). What advantages are offered by the complex regulation of YpkA involving multisite phosphorylation? It is conceivable that the requirement for multiple phosphorylation sites may impose a certain threshold of YpkA autophosphorylation that must be obtained for activation of YpkA. In this way, a low level of YpkA autophosphorylation would allow the kinase to phosphorylate its substrate(s), and thus, interfere with host signaling. Interestingly, YpkA is secreted in lower amounts relative to other Yersinia effectors (12). Thus, multisite phosphorylation could introduce the potential for sophisticated control over the dephosphorylation and inactivation of YpkA by host phosphatases and influence the strength and duration of YpkA kinase activity within the target cell. The multisite phosphorylation of proteins is indeed an extremely common mechanism for greatly increasing the regulatory potential of proteins. For example, the protein kinase MAPK-activated protein kinase-2 plays important roles in protecting cells against cell-damaging agents and infection. Its activation by stress-activated protein kinase-2 (SAPK2, also called p38) is accompanied by the phosphorylation of three residues, namely Thr-221, Ser-272, and Thr-334 (43). Phosphorylation of any one residue is insufficient for activation, whereas maximal activation is achieved if any two of the three sites are phosphorylated.

Despite the extensive analysis on YpkA, its mechanism of kinase activation remains enigmatic. Obviously, it is not clear how multisite phosphorylation orchestrates the activity of YpkA, and it will be of considerable interest to determine whether phosphorylation at each site is critical. Structural analysis of full-length YpkA would provide significant insights into the mechanism of YpkA kinase activation, as was reported for the C terminus of YpkA (32).

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