The Yersinia Protein Kinase A Is a Host Factor Inducible RhoA/Rac-binding Virulence Factor*

The pathogenic yersiniae inject proteins directly into eukaryotic cells that interfere with a number of cellular processes including phagocytosis and inflammatory-associated host responses. One of these injected proteins, the Yersinia protein kinase A (YpkA), has previously been shown to affect the morphology of cultured eukaryotic cells as well as to localize to the plasma membrane following its injection into HeLa cells. Here it is shown that these activities are mediated by separable domains of YpkA. The amino terminus, which contains the kinase domain, is sufficient to localize YpkA to the plasma membrane while the carboxyl terminus of YpkA is required for YpkAs morphological effects. YpkAs carboxyl-terminal region was found to affect the levels of actin-containing stress fibers as well as block the activation of the GTPase RhoA in Yersinia-infected cells. We show that the carboxyl-terminal region of YpkA, which contains sequences that bear similarity to the RhoA-binding domains of several eukaryotic RhoA-binding kinases, directly interacts with RhoA as well as Rac (but not Cdc42) and displays a slight but measurable binding preference for the GDP-bound form of RhoA. Surprisingly, YpkA binding to RhoAGTP affected neither the intrinsic nor guanine nucleotide exchange factor-mediated GDP/GTP exchange reaction suggesting that YpkA controls activated RhoA levels by a mechanism other than by simply blocking guanine nucleotide exchange factor activity. We go on to show that YpkAs kinase activity is neither dependent on nor promoted by its interaction with RhoA and Rac but is, however, entirely dependent on heat-sensitive eukaryotic factors present in HeLa cell extracts and fetal calf serum. Collectively, our data show that YpkA possesses both similarities and differences with the eukaryotic RhoA/Rac-binding kinases and suggest that the yersiniae utilize the Rho GTPases for unique activities during their interaction with eukaryotic cells.

Several Gram-negative bacteria species that live in close association, for at least part of their life cycle, with eukaryotic cells possess a “protein injection system” (designated as type III (Ref. 1)) that delivers bacterially encoded proteins directly into eukaryotic cells following attachment of the bacterium to the host cell (2). Effector proteins injected into eukaryotic cells by type III secretion systems have been shown to modulate a variety of processes such as Rhizobium-mediated nodulation (3), plant hypersensitivity responses (e.g. see Ref. 4), and animal immune responses (reviewed in Ref. 5). The type III-injected proteins of the pathogenic species of Yersinia inhibit eukaryotic defense responses that normally occur when cells contact bacteria such as phagocytosis (6) and the expression of pro-inflammatory cytokines (7–9). One such injected protein required for Yersinia-mediated lethality in the mouse, YpkA, is a 733-residue protein that contains in its NH2 terminus a domain that bears close sequence similarity to eukaryotic Ser/Thr protein kinases (10, 11). When injected into cultured eukaryotic cells by the Yersinia type III system YpkA induces cells to retract long finger-like extensions attached to the substratum (12).

The Rho GTPases (Rho, Rac, and Cdc42) form a subgroup of the Ras superfamily of small GTP-binding proteins that play a central regulatory role in a number of cellular activities which require cytoskeletal rearrangements such as phagocytosis and motility (13, 14). The importance of the Rho proteins in eukaryotic defense responses is indicated by the fact that a number of bacterial pathogens have evolved toxins that specifically target their activity. For example, pathogenic Escherichia coli, Bordetella bronchiseptica, and Clostridium botulinum produce toxins that either covalently modify RhoA in such a way that they remains “locked” in its active, GTP-bound state (E. coli (15, 16) and B. bronchiseptica (17)) or which inactivates RhoA by ADP-ribosylation (the C3 toxin of C. botulinum (18)). Similarly, the pathogenic Salmonella injects a protein toxin, SopE, into eukaryotic cells (19) that possesses a guanine nucleotide exchange factor (GEF)1 activity primarily for Cdc42 (20). SopEs activity within eukaryotic cells results in membrane ruffling which promotes Salmonella internalization (20). Interestingly, Salmonella injects a second protein, SptP, into eukaryotic cells that acts as a GTPase-activating protein toward Rac and Cdc42 which apparently serves to repair the host cell membrane following bacterial uptake (21). These examples illustrate that by targeting the small GTP-binding proteins bacterial pathogens are able to manipulate cellular processes to their advantage.

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; PCR, polymerase chain reaction; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; RBD, RhoA-binding domain; GFP, green fluorescent protein; GTPyS, guanosine 5′-O-(thiotriphosphate).
We were interested in identifying the domains of YpkA that are required for its morphological altering properties and its intracellular targeting to the plasma membrane. Additionally, based on comparative sequence analysis and how it affects eukaryotic cell morphology, we investigated whether YpkA targets the Rho proteins. We found that YpkA shares both similarities and differences with the eukaryotic RhoA-binding kinases perhaps indicating that ypkA ancestral gene, which was likely “captured” from a eukaryotic organism by the yersiniae at some point in the past, underwent extensive modification in order to serve the needs of a prokaryotic pathogen.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**For expression of ypkA in *Yersinia*, the multiple yop mutant strain (12) was transformed with either a plasmid (pEG2 (12)); here designated as pYpkA) containing the entire ypkA open reading frame and upstream promoter regions, or derivatives of pEG2 which contained either a point mutation in the kinase catalytic domain (ypkA1270A) or an internal deletion in the carboxyl terminus (ypkA432–628). pYpkA1270A was constructed by first synthesizing ypkA codons 1–284 by PCR using a 3′ primer containing the D270A substitution (underlined); 5′-CTCTCCGCATGCGCGTCAATACCATCTCCGGCTATGAGCTTACATGAATTCAGGTCTGATATCCCCAAT-3′. The resulting PCR product was digested with Bgl II and Nhe I (which in the wild type ypkA open reading frame at codons 66 and 281, respectively) and ligated into Bgl I/Nhe I-digested pYpkA. Presence of the point mutation was confirmed by sequencing. pYpkA432–628 was prepared simply by digesting pYpkA with Pst I followed by religation thus taking advantage of the fact that the two Pst I sites are in the same reading frame. RhoA-myc, GST-RhoA-, and GST-V14-RhoA-encoding plasmids were provided by Lawrence A. Quilliam (Indiana University School of Medicine) and Alan Hall (University College London, London, UK), respectively. A GST-SopE-encoding plasmid was provided by Edouard Galyov (Institute of Animal Health, United Kingdom).

A YpkA-encoding eukaryotic expression plasmid was constructed by first separately amplifying the 5′ and 3′ ends of ypkA using primer sets 5′-AACAGATTCATGAAAGCGGATTAATCGGAATGAGATGTCTCAGCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGACCTGCAATCCGCACTGCA
were detected by immunoblotting using RhoA-specific antisera (Santa Cruz, SC-418). YpkA does not detectably interact with RhoA in RIPA buffer.2

GST and GST-RhoA proteins were prepared by growing 25-ml cultures to A600 0.75, inducing with 0.01 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h, and lysing cells in a 1.2-ml volume of buffer N (1% Nonidet P-40, 150 mM NaCl, 10 mM MgCl2, 50 mM Tris, pH 8) by sonication. Cleared lysates were mixed with one-tenth volume (packed) buffer N-washed glutathione-Sepharose beads (Amersham Pharmacia Biochem) and incubated for 20 min at room temperature followed by two washings with buffer N. For nucleotide loading (25), beads were placed in 50 mM HEPES (pH 7), 50 mM NaCl, 5 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol with 0.5 mM GDP, GTP, or GTPγS (Roche Molecular Biochemicals) and incubated at 37 °C for 15 min. Nucleotide loading was terminated by adding MgCl2 to 20 mM and beads were collected and resuspended in either Yersinia- or HeLa-derived cell extracts.

Yersinia cell extracts were prepared by first diluting overnight cultures 1/20 into 25 ml of LB, incubating with shaking at 26 °C for 30 min, followed by a 60-min incubation at 37 °C which induces Yop expression (26). Cells were sonicated in 1.2 ml of cold buffer N, cleared by centrifugation, and supernatants were added directly to approximately 1 µg of either GST- or GST-RhoA-bound beads. For the recovery of YpkA from Yersinia-infected HeLa cells, overnight Yersinia cultures were diluted 10-fold into tissue culture media (RPMI supplemented with 10% fetal calf serum) and Yop expression induced as described above. Approximately 2 × 108 HeLa cells were infected in 10-cm Petri dishes at a multiplicity of infection of 100 in a 7.5-ml volume of antibiotic-free tissue culture media. After 2.5 h cells were washed twice with PBS and lysed on ice with 1 ml of buffer N containing protease inhibitors for 60 min. Buffer N extracts were then cleared by centrifugation (thus removing the insoluble cellular material as well as the bacteria which in buffer N are not lysed)2 and the resulting supernatants added directly to GST- or GST-RhoA-bound beads.

RhoA/YpkA Binding and Kinase Reactions—GST- or GST-GTPase-bound beads (containing approximately 1 µg of the various GST proteins) in a 30-µl packed volume were resuspended in YpkA-containing bacterial or HeLa cell extracts and rotated at 4 °C for 60 min. Beads were washed twice with cold buffer N and either resuspended directly in Laemmli sample buffer or 20 µl of premixed kinase buffer (buffer N containing 1 mM dithiothreitol, phosphatase inhibitor mixtures I and II (Sigma), 1 µl (10 µCi) of [γ-32P]ATP (Amersham Pharmacia Biotech) and either 5 µl of a buffer N extract prepared from uninfected HeLa cells (see above) or fetal calf serum. Some kinase reactions were supplemented with HeLa cell extract that had been incubated at 95 °C for 5 min. Following being shaken at room temperature for 15 min, beads were washed twice with buffer N and resuspended in Laemmli sample buffer and analyzed as described in the figure legend.

Immunoprecipitated YpkA was prepared by mixing 1 ml of YpkA-containing Yersinia buffer N extract with 20 µl of YpkA-specific antisera, incubating on ice for 60 min, followed by the addition of 100 µl (packed volume) of buffer N-washed protein G-Sepharose beads (Amersham Pharmacia Biotech). Samples were rotated 45 min at 4 °C, after which beads were washed twice with buffer N, divided into two equal parts, and resuspended in 100 µl of buffer N containing either GST or GST-RhoA that had been eluted from glutathione-Sepharose beads with 10 mM glutathione. Samples were rotated 30 min at 4 °C to allow RhoA/YpkA binding followed by the addition of 100 µl of 2 × kinase buffer supplemented with HeLa cell extract. After being shaken for 15 min at room temperature beads were washed twice with buffer N and analyzed as described in the figure legend.

GDP Dissociation Assay—Baculovirus stocks containing GST-Lbc and GST-Lac-encoding plasmids (27) were supplied by Judy Glaven (Harvard University) and amplified in SF-9 cells whereas Hi-5 cells were

2 K. Schesser, unpublished observations.

FIG. 2. Actin stress fibers in ypkA-transfected cells. HeLa cells were transiently transfected with plasmids encoding GFP (a marker for transfection) and full-length YpkA (top row), a variant of YpkA lacking the COOH terminus (middle row), or the empty vector (bottom row). Transfected cells were serum starved for 20 h and then fixed and stained with rhodamine-conjugated phalloidin and analyzed for either GFP (left column), rhodamine (middle column), or GFP/rhodamine (right column).
were washed and resuspended in buffer N containing 1 mM GTP and the same S-300 column used for the HeLa extract and eluted using above. For serum fractionation, 4 ml of fetal calf serum was applied to beads in kinase buffer. The kinase assay was performed as described (described above), incubating for 30 min at 4 °C, and resuspending the combining 1 ml of each fraction with bead-bound GST-RhoA/YpkA ml each. Fractions were assayed for their kinase promoting activity by of 20 ml/h. A prefraction was collected followed by 100 fractions of 2.5 column with a total volume of 340 ml and eluted with PBS at a flow-rate resulting supernatant was applied to a Sephacryl S-300-containing less than the YpkA signal from wt-YpkA-transfected cells indicating either lower expression or poorer recognition by the YpkA-specific antisera.

FIG. 3. Intracellular localization of YpkA in transfected cells. Twenty hours after transfection GFP expression was detected directly (green) while YpkA expression was analyzed by immunostaining (red). The images show a cut through the point in the x, y, and z axes (x-y, y-z, and x-z view). Note that YpkA is surrounding the intracellular expressed GFP. The YpkA signal from the ΔC-YpkA transfected cells was 3–4 times less than the YpkA signal from wt-YpkA-transfected cells indicating either lower expression or poorer recognition by the YpkA-specific antisera.

FIG. 4. Recovery of active RhoA from either uninfected or Yersinia-infected cells. Serum-starved HeLa cells were either left uninfected or infected with Yersinia expressing the indicated variants of YpkA for 80 min. Whole cell lysates were mixed with GST-rhotekinRBD-bound beads and the levels of RhoA recovered in the bead fraction were determined by immunoblotting (upper panel) as well as the levels of RhoA in the starting lysate (lower panel). The RBD of rhotekin is specific for GTP-bound RhoA.

used for protein expression. Insect cells were lysed by sonication in buffer N and GST proteins isolated similarly as bacterially expressed GST proteins. GST-RhoA-bound beads were loaded with [8-3H]GDP (12.9 Ci/mmol) as described above followed by saturable binding to YpkA (as determined by titration). GST-RhoA-GDP/YpkA-bound beads were washed and resuspended in buffer N containing 1 mM GTP and supplements (either 10 mM EDTA, or GST-Lbc, GST-Lac, or GST-SopE as indicated in the figure legend). Samples were incubated at 30 °C with occasional shaking and at the indicated time points aliquots were removed and added to a large excess of ice-cold buffer N. Beads were collected, washed a second time with cold buffer N, and resuspended in Scintillation fluid (OptiPhase HiSafe, Wallac Scintillation Products) and counted for 1 min.

RESULTS

Identifying Morphological and Localization Domains of YpkA—We first tested whether the kinase activity of YpkA was required for the YpkA-mediated effect on eukaryotic cell morphology (see Introduction). A kinase-deficient point mutant (D270A, see below) of YpkA was constructed and tested for its effects on HeLa cells. Surprisingly, the morphology of HeLa cells infected with Yersinia expressing the D270A variant of YpkA was indistinguishable from that of HeLa cells infected with wild type YpkA-expressing Yersinia (Fig. 1). In contrast, HeLa cells infected with Yersinia expressing a variant of YpkA with an internal deletion in its carboxyl-terminal region (YpkA543–640), did not induce the characteristic morphology even at very high inoculations (Fig. 1).

We next examined whether the YpkA-mediated morphological effect shown in Fig. 1 was accompanied by alterations in the actin-based cytoskeletal system. Approximately 1 h after the onset of infection, slightly fewer actin-containing stress fibers were observed in HeLa cells infected with Yersinia expressing full-length YpkA compared with cells infected with Yersinia expressing the YpkA543–640 variant (not shown). However, upon longer exposure (2 h) no stress fibers were observed in cells infected with Yersinia expressing either the full-length or Δ543–640 variants of YpkA as well as in cells infected with non-YpkA-expressing Yersinia strains (not shown). It is likely therefore that any YpkA-mediated effect on actin stress fibers is at least partially obscured by a general effect of the bacteria on the cytoskeletal system.

To determine whether YpkAs effect on cell morphology and actin dynamics was dependent on other Yersinia-derived factors, either full-length ypkA (both wild type and the D270A mutant) or a ypkA carboxyl-terminal deletion variant were cloned into a eukaryotic expression vector that also encoded gfp under the control of a separate viral promoter. The transfection of HeLa cells with the full-length YpkA expression plasmids
were notably less efficient (as determined by counting the number of GFP positive cells) compared with transfections using either the carboxyl-terminal deleted YpkA-encoding or the empty vector plasmids (not shown). *Yersinia*-expressed YpkA and YpkA expressed in transiently transfected HeLa cells were indistinguishable as analyzed by SDS-PAGE (not shown). Cells transfected with either the wild type or D270A ypkA variants displayed a morphology that was similar to that of cells infected with YpkA-expressing *Yersinia* (Fig. 2). In contrast, cells transfected with the carboxyl-terminal YpkA deletion mutant did not undergo the gross morphological alteration observed in full-length ypkA transfectants and appeared identical in their overall shape to cells transfected with the vector only (Fig. 2). Phalloidin staining indicated that while there were no differences in stress fibers between cells transfected with either the vector or the carboxyl-deleted YpkA-encoding plasmids, cells expressing full-length YpkA had a clear deficiency in the levels of stress fibers (Fig. 2).

We next addressed whether YpkAs localization to the plasma membrane was also, like its activity in affecting cellular morphology, dependent on domains located at the carboxyl terminus. By immunostaining it was found that transfected full-length YpkA localized to the plasma membrane (Fig. 3A). The carboxyl-terminal YpkA deletion mutant also accumulated at the plasma membrane (Fig. 3B) showing that the plasma membrane targeting domain is contained in the amino-terminal region of YpkA. It is worth noting that YpkA localized to the plasma membrane in both infected cells (12) as well as transfected cells which were not undergoing bacterial exposure thus indicating that YpkAs targeting to the membrane is not dependent on cellular stress responses that follow bacterial contact. Together these data show that transfected ypkA is expressed and that the activity of the protein within eukaryotic cells resembles that of bacterially injected YpkA. Furthermore, the YpkA properties of affecting cellular morphology and intracellular localization are mediated by separable domains located in the carboxyl- and amino-terminal regions, respectively.

**Recovery of RhoA<sub>TT</sub> from *Yersinia*-infected Cells**—Since the Rho GTPases are key regulators in controlling the actin-based cytoskeleton network, we determined whether YpkA affected the levels of RhoA<sub>TT</sub> in *Yersinia*-infected cells. Using a RhoA<sub>TT</sub>-specific affinity purification assay (24) we observed a slight increase in the amount of RhoA<sub>TT</sub> recovered in cells infected with *Yersinia* expressing either the wild type or D270A variants of YpkA compared with the amount of RhoA<sub>TT</sub> recovered in uninfected cells (Fig. 4). However, significantly more RhoA<sub>TTP</sub> was recovered in cells infected with *Yersinia* expressing the Δ543–640 variant of YpkA compared with either the uninfected cells or the cells infected with *Yersinia* expressing full-length YpkA variants (compare lanes 2 with 1, 3, and 4). These data indicate that under these infection and assay conditions the carboxyl-terminal domain of YpkA affects the levels of active RhoA<sub>TT</sub>, in *Yersinia*-infected eukaryotic cells.

**YpkA/Rho GTPase Interaction Assays**—Recently the structure of the RhoA-binding domain (RBD) of PKN/PRK1 complexed with RhoA was reported (28). Within its carboxyl terminus region YpkA possesses four pairs of predicted α helices that each bear sequence similarity to the RhoA-binding helices of PKN/PRK1 and other RhoA-binding kinases (Fig. 5). Therefore we tested whether YpkA binds RhoA in yeast two-hybrid and GST pull-down assays. A Gal4<sub>BD</sub>-YpkA<sub>442–733</sub> hybrid protein, containing all four pairs of the predicted RhoA-interacting helices, tested positive for interaction with a Gal4<sub>AD</sub>-RhoA hybrid protein in a yeast two-hybrid assay (Fig. 6). In contrast, yeast containing plasmids encoding Gal4<sub>BD</sub>-RhoA and either Gal4<sub>BD</sub>-YpkA<sub>543–640</sub> or Gal4<sub>BD</sub>-YpkA<sub>97–347</sub> did not express the reporter gene (Fig. 6A). (The Δ543–640 YpkA variant is missing three helices in the middle of YpkAs RBD.) To determine whether the Gal4<sub>BD</sub>-YpkA<sub>442–733</sub>/Gal4<sub>AD</sub>-RhoA-dependent reporter gene expression is affected by coexpression of either YpkA or RhoA, a “competitive” two-hybrid assay was performed (29). Yeast were transformed with plasmids encoding Gal4<sub>BD</sub>-YpkA<sub>442–733</sub>, Gal4<sub>AD</sub>-RhoA, and either YpkA<sub>449–733</sub> or RhoA. Transformants expressing either of the competitors (i.e., YpkA<sub>449–733</sub> or RhoA) were unable to grow in the absence of histidine (Fig. 6B) indicating that the competitors were able to decrease the level of interaction between Gal4<sub>BD</sub>-YpkA<sub>442–733</sub> and Gal4<sub>AD</sub>-RhoA.

To further confirm the YpkA-RhoA association, we mixed YpkA-containing *Yersinia* extracts with bead-bound GST or GST-RhoA and assayed for YpkA in the bead-bound fraction by immunoblotting. Full-length YpkA copurified with GST-RhoA but not with GST (Fig. 7A, lanes 1–3). YpkA containing a point mutation in the kinase catalytic domain (D270A) (see below) copurified with GST-RhoA (lanes 5–7) whereas a YpkA variant with an internal deletion in the carboxyl-terminal region (YpkA<sub>543–640</sub>) did not associate with GST-RhoA at detectable levels (lanes 8–10). These data show that full-length YpkA associates with RhoA and, similar to what was observed in the two-hybrid assay (Fig. 6), the carboxyl-terminal region of YpkA is required for this interaction to occur. Despite several attempts endogenous RhoA was not detected in YpkA immunoprecipitates prepared from infected HeLa cells.

Nearly all the eukaryotic RhoA-binding kinases described to...
date bind only RhoA and not the closely related Rac and Cdc42 GTPases. To determine whether YpkA binds the other members of the Rho family, we performed an experiment similar to the one shown in Fig. 7A using GST-RhoA, -Rac, and -Cdc42 hybrid proteins. YpkA bound RhoA and Rac with similar affinities (Fig. 7B). In contrast, relatively much lower levels of YpkA copurified with the GST-Cdc42-bound beads. These data indicate that YpkAs binding affinities for the Rho GTPases is unique among RhoA-binding kinases.

Characterization of the YpkA/RhoA Interaction—All but one of the eukaryotic RhoA-binding kinases described to date preferentially or exclusively bind RhoA in its active, GTP-bound state in both yeast two-hybrid and in vitro binding assays. To test whether YpkA displays similar binding characteristics, yeast were co-transformed with plasmids encoding Gal4BD-YpkA442–733 and Gal4AD-V14-RhoA (the constitutively active form of RhoA). No growth of the Gal4BD-YpkA438–733/Gal4AD-V14-RhoA co-transformants were observed under conditions selecting for expression of the ADE2 or HIS3 reporter genes (not shown) indicating that, unlike, for example, p160lck (30), YpkA does not detectably interact with the constitutively active form of RhoA in the yeast two-hybrid assay. Likewise, barely detectable levels of full-length YpkA copurified with GST-V14-RhoA in the GST pull-down assay (Fig. 7A, lane 4).

As a more direct test to determine whether YpkA displays nucleotide-dependent RhoA binding, we performed GST pull-down assays using GST-RhoA loaded with GDP, GTP, or GTP\(\gamma\)S. (In the experiments shown in Fig. 7, A and B, E. coli-produced GST-RhoA, -Rac, and -Cdc42, which are primarily in the GDP-bound form, were used directly in the binding assay.) Using a YpkA-containing Yersinia extract, there was approximately a 3-fold greater amount of YpkA that copurified with GST-RhoA\(\gamma\)GDP compared with the amount of YpkA that copurified with either GST-RhoA\(\gamma\)GTP or GST-RhoA\(\gamma\)GTP\(\gamma\)S (Fig. 7C, upper panel). Preferential YpkA binding to GDP-bound RhoA was observed over an 80-fold range of input YpkA (Fig. 7D).

To test the RhoA binding activity of YpkA that had been injected into eukaryotic cells, Yersinia-infected HeLa cell extracts were prepared using conditions that fractionated injected YpkA and bacterially associated YpkA (see “Experimental Procedures”). Injected YpkA displayed an identical pattern of RhoA binding as that found in bacterial extracts (Fig. 7C, lower panel) implying that YpkA does not undergo modifications following its injection into eukaryotic cells that affect its association with RhoA. Taken together, these data show that YpkA binds both the active and inactive forms of RhoA and Rac with, in the case of RhoA, a slight but measurable greater

(4–6). Bait plasmids contained YpkA residues 442–733 (1 and 4), 442–733 Δ543–640 (2 and 5), or residues 97–347 (3 and 6), the latter encompassing the kinase domain. Transformant yeast strains were plated on minimal media containing adenine and histidine (top), or lacking adenine (bottom) or histidine (not shown; results were identical to that shown for minus adenine). Growth in the absence of either adenine or histidine required expression of the ADE2 or HIS3 reporter genes, respectively. B, yeast were co-transformed with Gal4AD-RhoA-encoding plasmids and Gal4BD-YpkA442–733-encoding bait plasmids which additionally encoded, at a second multiple cloning site and under the control of the inducible Met25 promoter, competitors which were RhoA (1), empty (2), or YpkA449–733 (3). Transformants were plated on minimal media lacking histidine and either containing methionine (non-inducing) or lacking methionine (inducing).
affinity for the GDP-bound form of RhoA.

RhoA GDP Dissociation Assays—One possible mechanism by which YpkA may affect RhoA activation in Yersinia-infected cells (Fig. 4) is by blocking the activity of the GEFs. Therefore we tested whether YpkA affected the activation of RhoA by measuring the rate of GDP dissociation from either free or YpkA-bound RhoA_{GDP}. The intrinsic rate of GDP dissociation from RhoA_{GDP} did not appear to be affected by YpkA (Fig. 8). Surprisingly, YpkA also did not affect the ability of the RhoA-specific GEFs Lbc and Lsc (27) or the Salmonella-encoded GEF SopE (Ref. 20; see Introduction) to promote the rate of GDP dissociation from RhoA_{GDP} (Fig. 8). Identical results were obtained when the assays were performed in the presence of eukaryotic cytosolic extracts (not shown) under conditions in which YpkA is known to physically interact with a kinase-promoting cofactor (see below). These data indicate that YpkA binds RhoA in such a way as to keep the latter in an “activatable” conformation and furthermore, suggests that YpkA affects RhoA_{GTP} levels in cells by some other mechanism than by simply blocking GEF activity.

RhoA Binding and YpkA Kinase Activity—To test whether YpkA possesses enzymatic activity while bound to RhoA, GST-RhoA-YpkA-loaded beads were resuspended in a kinase buffer containing [γ-32P]ATP. YpkA autophosphorylation was assayed since the natural substrates of YpkA are unknown. No phosphorylation of YpkA was observed in kinase buffer alone (Fig. 9A, lane 1). However, YpkA was phosphorylated if the kinase buffer was supplemented with a HeLa cell detergent extract (compare lanes 1 and 3) whereas no YpkA phosphorylation was observed in kinase reactions supplemented with detergent extracts prepared from Yersinia cells (not shown). The kinase promoting activity in HeLa cell extracts was heat-sensitive (lane 7) and dose-dependent (Fig. 9D). Labeling of YpkA was also dependent on a catalytically active YpkA (compare lanes 3 and 5) indicating that the phosphorylation observed in lane 3 was due to autophosphorylation (or possibly trans-phosphorylation) and not to kinases present in the HeLa cell extract. Autophosphorylation of Rac-bound YpkA was also enhanced by HeLa cell extract (Fig. 9C). These data show that YpkA phosphorylation occurs while YpkA is bound to RhoA and Rac and that this reaction is dependent on a eukaryotically derived cofactor. RhoA has been reported to be phosphorylated by protein kinase A that negatively regulates its activity (31). No phosphorylation of GST-RhoA by YpkA was observed (Fig. 9A).

The enzymatic activities of several RhoA- and Rac-binding kinases have been shown to be affected (although in some cases modestly) by GTPase binding (for examples, see Refs. 30 and 32). During the kinase assay shown in Fig. 9A, a fraction of YpkA became dissociated from the RhoA-bound beads and although small in amount (as judged by the immunoblot), this fraction of YpkA was highly phosphorylated (lane 4). The level of dissociation of YpkA from RhoA during the kinase assay was not detectably affected by YpkA phosphorylation since approximately equal amounts of YpkA dissociated in samples in which YpkA did not become phosphorylated (lanes 2, 6, and 8). Assuming that the immunoblots can reveal small changes in YpkA levels, these data suggest that while YpkA can undergo autophosphorylation while bound to RhoA, its level of phosphorylation increases following its dissociation with RhoA.

To test whether the enzymatic activity of immunoglobulin-immobilized YpkA is affected by RhoA, YpkA autophosphorylation was assayed in the presence of either GST or GST-RhoA. Yersinia-expressed YpkA was immunoprecipitated, incubated with either soluble GST or GST-RhoA, and then placed in a kinase reaction supplemented with a HeLa detergent cell ex-

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**Fig. 7. GST pull-down assays.** A, cytoplasmic extracts prepared from Yersinia strains expressing wild type YpkA (lane 1), a YpkA kinase point mutant (lane 5), or YpkA with an internal deletion of residues 543–640 (lane 8), were mixed with glutathione beads bound with GST (lanes 2, 6, and 9), GST-RhoA (lanes 3, 7, and 10), or GST-Val^{14}-RhoA (lane 4). After a 1-h incubation beads were washed, boiled in Laemmli buffer, and subjected to SDS-PAGE. YpkA levels of the starting lysates or in the bead-bound fraction were determined by immunoblotting using YpkA-specific antisera. B, YpkA-containing lysate (lane 1) was mixed with glutathione beads bound with GST, GST-RhoA, GST-Rac, or GST-Cdc42 and processed as described in A. Blotted proteins were analyzed for either YpkA (top and middle panels) or GST (lower panel) by immunoblotting. Shown in the middle panel is a longer exposure of the autoradiogram shown in the top panel. C, cytoplasmic extracts prepared from either Yersinia cultures (upper panel) or from Yersinia-infected HeLa cells (lower panel) were mixed with bead-bound GST-RhoA preloaded with GDP (lane 2), GTP (lane 3), or GTPγS (lane 4). Beads and bead-associated YpkA were processed and analyzed as in A. Some degradation of YpkA recovered from infected HeLa cells was observed in the experiment shown. A 10-, 3-, and 2-fold dilution of the GST-RhoA_{GDP} sample (lane 2) were run in lanes 5–7 for comparative purposes. D, a constant amount of either bead-bound GST-RhoA_{GDP} or GST-RhoA_{GTP} were mixed with various amounts of Yersinia-expressed YpkA and processed and analyzed as in A.
tract. Although immunoprecipitated YpkA was activated by the HeLa cell-derived cofactor the levels of YpkA autophosphorylation did not appear to be affected by RhoA (Fig. 9B). Additionally we tested whether there were differences in YpkA’s kinase activity when bound to either GDP- or GTPγS-bound RhoA or Rac. There were no apparent differences in kinase activity observed between RhoAGDP-/RacGDP-bound YpkA and RhoAGTPγS/RacGTPγS-bound YpkA (Fig. 9C). Taken together, these data indicate that RhoA does not enhance and in fact may be detrimental (perhaps by steric interference) to YpkA’s autophosphorylation activity.

**Eukaryotic Cofactors**—Since YpkA is thought to function exclusively inside eukaryotic cells following its injection by the Yersinia type III secretion system, we were surprised to find that, like extracts prepared from HeLa cells, fetal calf serum also contained a dose-dependent kinase promoting activity (Fig. 9D) that was heat-sensitive (not shown). The kinase promoting activity of both HeLa cell extracts and serum were not lost following dialysis using membranes with a 12–14-kDa cut off further showing, along with the heat sensitivity of these activities, that ions or small nonpeptide molecules are not activating YpkA. To determine whether the kinase-promoting factors present in HeLa cell extracts and serum were not lost following dialysis using membranes with a 12–14-kDa cut off further showing, along with the heat sensitivity of these activities, that ions or small nonpeptide molecules are not activating YpkA. To determine whether the kinase-promoting factors present in HeLa cell extracts and serum were similar to one another, we subjected a cytosolic HeLa cell extract and serum to size-exclusion chromatography and tested the resulting fractions for kinase promoting activity (Fig. 9D) that was heat-sensitive (not shown). The kinase promoting activity of both HeLa cell extracts and serum were not lost following dialysis using membranes with a 12–14-kDa cut off further showing, along with the heat sensitivity of these activities, that ions or small nonpeptide molecules are not activating YpkA. To determine whether the kinase-promoting factors present in HeLa cell extracts and serum were similar to one another, we subjected a cytosolic HeLa cell extract and serum to size-exclusion chromatography and tested the resulting fractions for kinase promoting activity. Bead-bound GST-RhoA/YpkA was incubated with various HeLa cell fractions in a binding reaction after which the washed beads were resuspended in kinase buffer containing [γ-32P]ATP. The highest kinase promoting activity was present in the same fractions as molecules with hydrodynamic sizes of 40–50 kDa (Fig. 10). These data show that the kinase promoting activity can be “fished” out of the HeLa cell fractions indicating that kinase activation likely involves a relatively stable physical interaction between the factor and YpkA and that this interaction is not impeded by RhoA.

Unlike the HeLa cell fractions, the serum fractions had higher protein levels and could be used directly in a kinase reaction. The serum kinase promoting activity was found in fractions containing molecules with hydrodynamic sizes between 100 and 300 kDa (Fig. 10). The activity was found in a peak that was less distinct, but clearly eluted from the column earlier than the HeLa cell-derived factor. Like the activity from HeLa cells, the kinase promoting activity could be fished out of active, but not inactive, serum fractions (not shown). Taken together these data show that YpkA can be enzymatically activated by different eukaryotic factors and that this activation likely involves a physical interaction that is distinct from, and not affected by the RhoA interaction.

**DISCUSSION**

Recently it has been shown that YopJ, which is encoded by the same operon as YpkA, physically interacts with several members of the mitogen-activated protein kinase family as well as IKKβ (33). The targeting of mitogen-activated protein kinases/IKKβ by YopJ inhibits the signaling activities of these proteins and likely accounts for the YopJ-dependent suppression of tumor necrosis factor-α and interleukin-8 expression in Yersinia-infected macrophages and epithelial-like cells (34–36). Similarly, we show here that YpkA physically interacts with the eukaryotic regulatory proteins RhoA and Rac which results in decreased levels of activated RhoA and an alteration of morphology in cells infected with YpkA-expressing Yersinia. Additionally, our analysis of the YpkA/RhoA interaction revealed that YpkA displays both similarities and differences with the eukaryotic RhoA-binding kinases.

Sequence comparison indicates that YpkA may possess up to four separate RBDs although it remains to be determined

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**Fig. 8. GDP dissociation assays.** Either unbound (A and C) or YpkA-bound (B and D) GST-RhoA[8-3H]GDP-loaded glutathione beads (approximately 1 μg of protein) were incubated at 30 °C with the indicated supplements. At the indicated time points aliquots were removed and the aliquoted beads were washed and resuspended in scintillation fluid and counted for 1 min. The amount of supplements added were: GST-Lbc and GST-Lac, X = 100 ng; GST-SopE, 800 ng; EDTA, 10 μl. The number in parentheses represents the fraction of counts/min remaining in the 60-min sample compared with the counts/min in the starting sample.
whether each of these potential domains individually bind RhoA. All but one of the described RBDs possessed by eukaryotic kinases have been shown to have a strong preference for the GTP-bound RhoA. The one exception, ACC2/HR1bPRK1 of PKN/PRK1, binds, similar to what we have found for YpkA, both the GDP- and GTP-bound forms of RhoA (37). Interestingly, PKN/PRK1 possesses a second RBD, ACC1/HR1aPRK1, which exclusively binds the GTP-bound form of RhoA (37). There are no obvious sequence differences between these two RBDs (see Fig. 5) which could account for their differing specificities. Likewise, YpkA does not contain sequences bearing obvious similarities to any of the Rac-binding kinases. Our data does not preclude the possibility that YpkAs RhoA- and Rac-binding domains are different. In any case the fact that YpkA

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**Fig. 9.** YpkA kinase activity. A, GST-RhoA-loaded beads were mixed with cytoplasmic extracts prepared from Yersinia strains expressing wild type YpkA (lanes 1–4, 7, and 8), the D270A point mutant (lanes 5 and 6), or no YpkA (lanes 9 and 10). Following the binding reaction beads were resuspended in [γ-32P]ATP-containing kinase buffer either alone (−) or supplemented with untreated (+) or heat-treated (HT) HeLa cell detergent extract. Following incubation in kinase buffer, 25% of bead-bound (B) and supernatant fractions (S) were subjected to SDS-PAGE and either analyzed directly for 32P-incorporation (top) or immunoblotted using YpkA-specific antisera (bottom). B, immunoprecipitated YpkA was incubated in kinase reaction buffer supplemented with HeLa cell detergent extract in the presence of either soluble GST or GST-RhoA. Following the reaction, beads were washed and analyzed as in A. Two different amounts of each sample (4X and X) were loaded for comparative purposes. C, GST-RhoA- and GST-Rac-bound glutathione beads were loaded with the indicated nucleotide, incubated with YpkA-containing Yersinia extract, and resuspended in kinase buffer either alone (−) or in kinase buffer supplemented with HeLa cell detergent extract (+). Analysis of YpkA phosphorylation (top panel) and binding (lower panel) were performed as in A. D, GST-RhoA/ YpkA-bound beads were resuspended in either nonsupplemented kinase buffer (lane 1) or kinase buffer supplemented with increasing amounts of either a HeLa cell detergent extract (lanes 2–4) or fetal calf serum (lanes 5–7) and were processed and analyzed as in A.

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**Fig. 10.** Chromatographic properties of the HeLa- and serum-derived YpkA-inducing factors. Either a 100,000 × g cytosolic HeLa cell extract or untreated fetal calf serum were fractionated using Sephacryl S-300. Fractions were measured for protein content (left Y axis) and used in a YpkA kinase assay (inset, 32P shown on top and YpkA immunoblot shown on bottom). HeLa cell fractions were mixed with bead-bound GST-RhoA/YpkA, incubated for 30 min at 4 °C, and then collected and resuspended in [γ-32P]ATP-containing kinase buffer and analyzed as in Fig. 7A. Serum fractions were added directly to the kinase reaction as in Fig. 7A. The 32P signal was quantified by a luminescent image analyzer (Fujifilm) and reported relative to the lowest signal (bars, right Y axis). The Yersinia Protein Kinase A

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The Yersinia Protein Kinase A
interacts with GDP- and GTP-bound Rac in addition to RhoA makes it unique among the RhoA-binding kinases which, except for one example (25), exclusively bind RhoA.

How is YpkA’s carboxyl-terminal located RhoA/Rac-binding domain related to the amino-terminal located kinase domain? In this study we show that YpkA’s RhoA-binding and enzymatic activities appear to be independent of one another. YpkA’s RhoA-independent kinase activity sets it apart from other RhoA-binding kinases in which RhoA binding appears to modulate, either positively or negatively, enzymatic activity (30, 32, 38). RhoA binding is also thought to function to localize RhoA-binding kinases to the plasma membrane apparently in order to place them near their substrates (39–41). Here we clearly show that the RhoA-binding domain of YpkA is not required to translocate YpkA to the plasma membrane but rather that the membrane localization domain is located in YpkA’s amino terminus. Thus it remains to be determined the relationship, if any, between YpkA’s RhoA/Rac binding and kinase activities.

Although YpkA is not dependent on RhoA to function as a kinase in vitro, YpkA does require eukaryotic factors that are present in HeLa cell cytosolic extracts and fetal calf serum. Based on our data the YpkA-inducing factors present in HeLa cells and serum are almost certainly these. These factors directly interact with YpkA independently of RhoA. It remains to be determined the identity of the kinase-promoting factors and the nature of their interaction with YpkA. Host factor activation of YpkA may be a strategy by which Yersinia prevents YpkA from being active within the bacterial cell. This strategy is utilized by pathogens such as Bordetella pertussis and Pseudomonas aeruginosa which express a calmodulin-activated adenylate cyclase (Cya) and a 14-3-3-activated Ras and activated adenylate cyclase (Cya) and a 14-3-3-activated Ras.

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