

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

Received for publication, April 7, 2000, and in revised form, August 8, 2000
Published, JBC Papers in Press, August 18, 2000, DOI 10.1074/jbc.M003009200

Jean-Marie Dukuzumuremyi‡, Roland Rosqvist§, Bengt Hallberg§, Bo Åkerström¶, Hans Wolf-Watz§, and Kurt Schesser‡||

From the ‡Department of Cell & Molecular Biology, Immunology Section, Lund University, SE-223 62 Lund, Sweden, the §Department of Cell & Molecular Biology, Umeå University, SE-901 87 Umeå, Sweden, and the ¶Department of Cell & Molecular Biology, Molecular Signalling Section, Lund University, SE-221 00 Lund, Sweden

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 YpkA's morphological effects. YpkA's 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 RhoA_{GDP} 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 YpkA's 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

* This work was supported by the Swedish Institute and the Swedish Medical and Natural Research Councils (to H. W.-W.), the Swedish Cancer Society and the Swedish Foundation of Strategic Research (to H. W.-W.), and the Fogarty International Center (NIH), Active Biotech (Lund, Sweden), the Crafoordska Stiftelsen, and the Greta and Johan Kocks Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

|| To whom correspondence should be addressed: CMB/Immunology Section, Sölvegatan 21, Lund University, SE-223 62 Lund, Sweden. Tel.: 46-46-222-0313; Fax: 46-46-222-4218; E-mail: kurt.schesser@immuno.lu.se.

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 root 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 leaving 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 it 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). SopE's 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.

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; GTPγS, 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*s 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 (pYpkA_{D270A}) or an internal deletion in the carboxyl terminus (pYpkA_{Δ543-640}). pYpkA_{D270A} was constructed by first synthesizing *ypkA* codons 1–284 by PCR using a 3' primer containing the D270A substitution (underlined): 5'-CTCTCCGCTAGCGCGGTCAATACCA-CATTACCGGGTTTGATAGCGTTATGTAC-3'. The resulting PCR product was digested with *Bgl*II and *Nhe*I (which appear in the wild type *ypkA* open reading frame at codons 66 and 281, respectively) and ligated into *Bgl*II/*Nhe*I-digested pYpkA. Presence of the point mutation was confirmed by sequencing. pYpkA_{Δ543-640} 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 Brahim Houssa and W. J. van Blitterswijk (The Netherlands Cancer Institute, Amsterdam). The GST-Rac1- and GST-Cdc42-encoding plasmids were obtained from 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'-AACAAAGATCCATATGAAAAGCGTGAATAATCATGGGAAC-3'/5'-CTCCCGAAGCTTCTTGGGTGT-3' and 5'-CCCAAGAAGCTTCGGGA-GCTA-3'/5'-TTACGCCGCGCTCATCCAT-3', respectively. The respective PCR products were cloned into the *Sma*I site of pGEM-3 (Promega) and sequenced. The 5' and 3' *ypkA* fragments were excised from pGEM-3 with either *Pst*I/*Hind*III or *Hind*III/*Hinc*II, respectively, ligated to each other (the *Hind*III site appears naturally in *ypkA* at codon 444), and ligated back into pGEM-3 yielding the plasmid pGEM-*ypkA*. Full-length *ypkA* was then excised from pGEM-3 with *Xba*I and cloned into the corresponding site of pCMS-EGFP, that encodes GFP from a separate cytomegalovirus promoter (CLONTECH). A D270A YpkA variant cloned in pCMS-EGFP was made by first digesting pYpkA_{D270A} (see above) with *Bgl*II/*Nhe*I, isolating the resulting 600-base pair fragment (that contains the D270A substitution), and using this fragment to replace the corresponding *Bgl*II/*Nhe*I fragment from pGEM-*ypkA* (see above) yielding the plasmid pGEM-*ypkA*_{D270A}. pGEM-*ypkA*_{D270A} was digested with *Xba*I and cloned into the corresponding site of pCMS-EGFP. A COOH-terminal deletion *ypkA* variant was constructed by digesting pGEM/YpkA with *Hind*III, filling with Klenow, followed by digestion with *Eco*RI. The resulting 1.3-kilobase fragment, encoding the 443 NH₂-terminal codons of *ypkA*, was ligated into pCMS-EGFP that had been digested with *Xba*I, filled with Klenow, followed by digestion with *Eco*RI.

The yeast two-hybrid plasmids pGal4_{BD}-YpkA₉₇₋₃₄₇ and pGal4_{BD}-YpkA₄₄₂₋₇₃₃ were constructed by amplifying *ypkA* using 5'-CGCAG-GAATTCAGTCTGATATCCCAAT-3' and 5'-GAATTCAGTCTG-CAGTAGGCTTTATC-3' for codons 97–347 and 5'-GTAAGGCAGATC-TCACCAAGAAGCTTCGGGAG-3' and 5'-TTGGATGCATTCACATC-CATTCCCGCTCCAACCGTT-3' for codons 442–733 and ligating the resulting digested PCR products into pGBT9 (CLONTECH). pGal4_{BD}-YpkA₄₄₂₋₇₃₃ was prepared by digesting pGal4_{BD}-YpkA₄₄₂₋₇₃₃ with *Pst*I followed by religation (see above). DNA fragments encoding human RhoA codons 1–719 were amplified by PCR from a cDNA library prepared from HeLa cells using the downstream primer 5'-ATTCATA-GATCTTTGCAGAGCAGCTCTCGTAGC-3' with either a wild type encoding upstream primer (5'-GAGATCGAATTCATGGCTGCCATCCGG-AAGAAA-3') or an upstream primer containing a G14V substitution

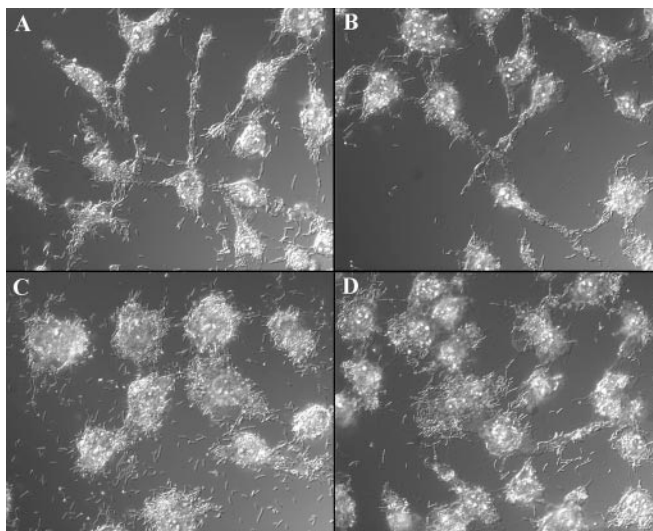


FIG. 1. YpkA-mediated effects on cell morphology. HeLa cells were cultured on glass slides, infected with *Y. pseudotuberculosis*, and visualized by differential interference contrast microscopy 2 h postinfection. Cells were infected with multiple yop mutant (MYM) *Yersinia* strain expressing either wild type YpkA (A), the catalytically inactive YpkA_{D270A} variant of YpkA (B), or a variant of YpkA, YpkA_{Δ543-640}, containing an internal deletion in the COOH terminus (C). D, cells infected with the multiple yop mutant host strain. Note the pronounced retraction fibers in A and B.

(underlined) (5'-GAGATCGAATTCATGGCTGCCATCCGGAAGAAAC-TGGTGATTGTTGGTGTAGCTAGCCTGT-3'). The RhoA-encoding PCR products were cloned into pGAD424 (CLONTECH). The yeast host strain used for the two-hybrid assay was PJ69-4A (22).

For the competitive two-hybrid assay, the *Sma*I/*Xba*I fragment of pBridge (CLONTECH), a pGBT9-derived bait plasmid possessing a second multiple cloning site (MCSII) located downstream of the inducible *Met25* promoter, was replaced by the corresponding *Sma*I/*Xba*I fragment excised from pGal4_{BD}-YpkA₄₄₂₋₇₃₃ (see above) which contained the *ypkA* open reading frame. To clone the YpkA or RhoA "competitors" into MCSII, YpkA- or RhoA-encoding fragments were generated by PCR using the following primer sets: YpkA₄₄₉₋₇₃₃, 5'-ATTGCGGCCGCATCTGATTTGGCTTAGGACGCATTTG-3' and 5'-AT-TAGATCTTCACATCCATCCCGCTCGTT-3'; RhoA, 5'-ATTGCGGCC-GCAATGGCTGCCATCCGGAAGAAA-3' and 5'-ATTCATAGATCTTT-GCAGAGCAGCTCTCGTAGC-3'. The yeast host strain used for the competitive two-hybrid assay was CG-1945 (CLONTECH).

Confocal Microscopy and Image Processing—Cells were transfected using LipofectAMINE (Life Technologies, Inc.), fixed in 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and processed for indirect immunofluorescence labeling (for details see Ref. 23) using affinity purified rabbit anti-YpkA antibodies followed by Alexa 488-conjugated goat anti-rabbit antibodies (Molecular Probes, Eugene, OR). The specimens were analyzed using a laser scanning confocal microscope equipped with dual detectors and an argon-krypton laser for simultaneous scanning of two different fluorochromes (Multiprobe 2001, Molecular Dynamics, Sunnyvale, CA). Sets of fluorescent images were acquired simultaneously for GFP and Alexa 488-tagged markers. Companion images (30 sections with an image size of 512 × 512) were scanned with 0.07- μ m pixel size and 0.37- μ m step size. The acquired sets of images were processed using the Huygens2 deconvolution algorithm (Bitplane) and then volume rendered using the Imaris (Bitplane) software. For actin staining transfected cells were fixed and permeabilized, incubated with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 30 min at 37 °C, and analyzed similarly as immunostained cells.

Protein Extractions—The affinity purification of active RhoA was performed essential as described by Ren *et al.* (24). HeLa cells were infected as described below after which they were washed with cold PBS and lysed in RIPA buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂) supplemented with 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml each of leupeptin, pepstatin, and aprotinin. Lysates were cleared by centrifugation and equivalent amounts of lysate proteins were incubated with the GST-rhotekin_{RBD} protein for 1 h. After four washes in lysis buffer samples were boiled in Laemmli sample buffer and bound Rho proteins

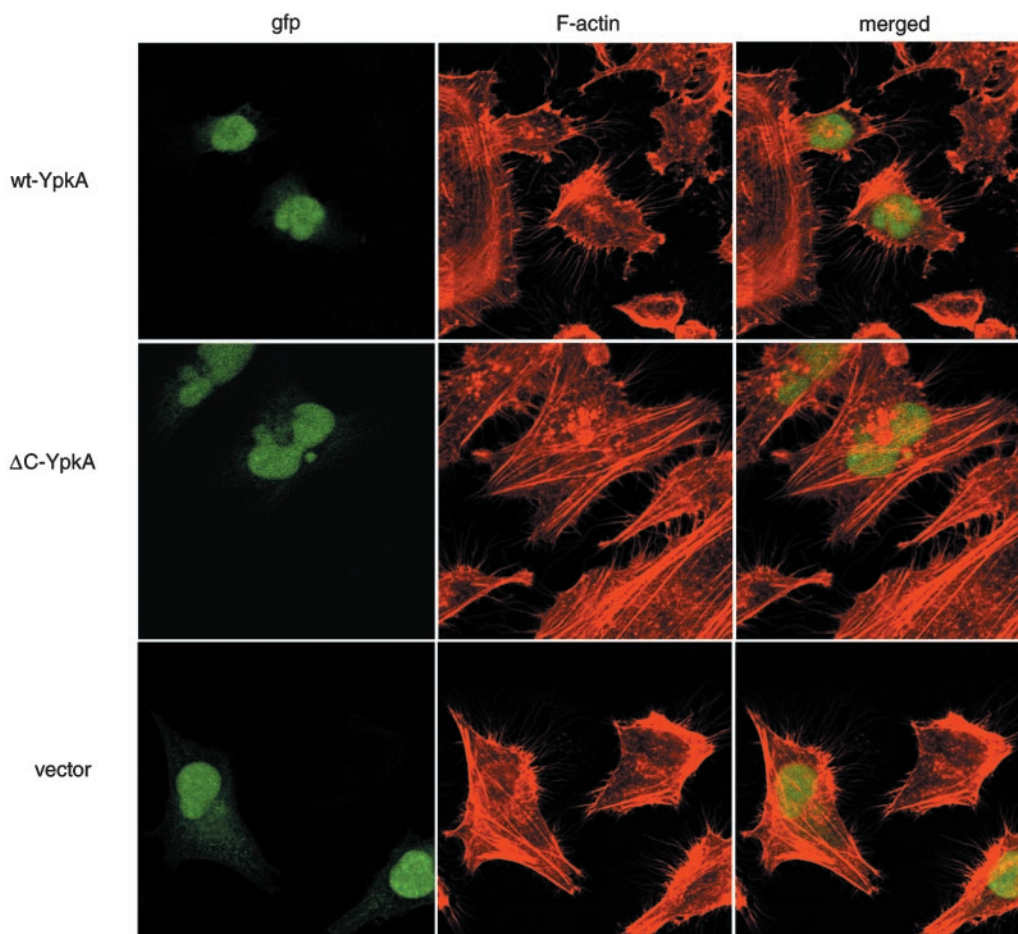


FIG. 2. Actin stress fibers in *ypkA*-transfected cells. HeLa cells were transiently transfected with plasmids encoding GFP (a marker for transfection) and full-length YpkA (top row), a variant of YpkA lacking the COOH terminus (middle row), or the empty vector (bottom row). Transfected cells were serum 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 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 A_{600} 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 GTP γ S (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 μ g 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 2×10^6 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 re-

moving 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 μ g of the various GST proteins) in a 30- μ l 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 μ l of premixed kinase buffer (buffer N containing 1 mM dithiothreitol, phosphatase inhibitor mixtures I and II (Sigma), 1 μ l (10 μ Ci) of [γ -³²P]ATP (Amersham Pharmacia Biotech) and either 5 μ l 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 μ l of YpkA-specific antisera, incubating on ice for 60 min, followed by the addition of 100 μ l (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 μ l 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 μ l of $2 \times$ 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-Lsc-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.

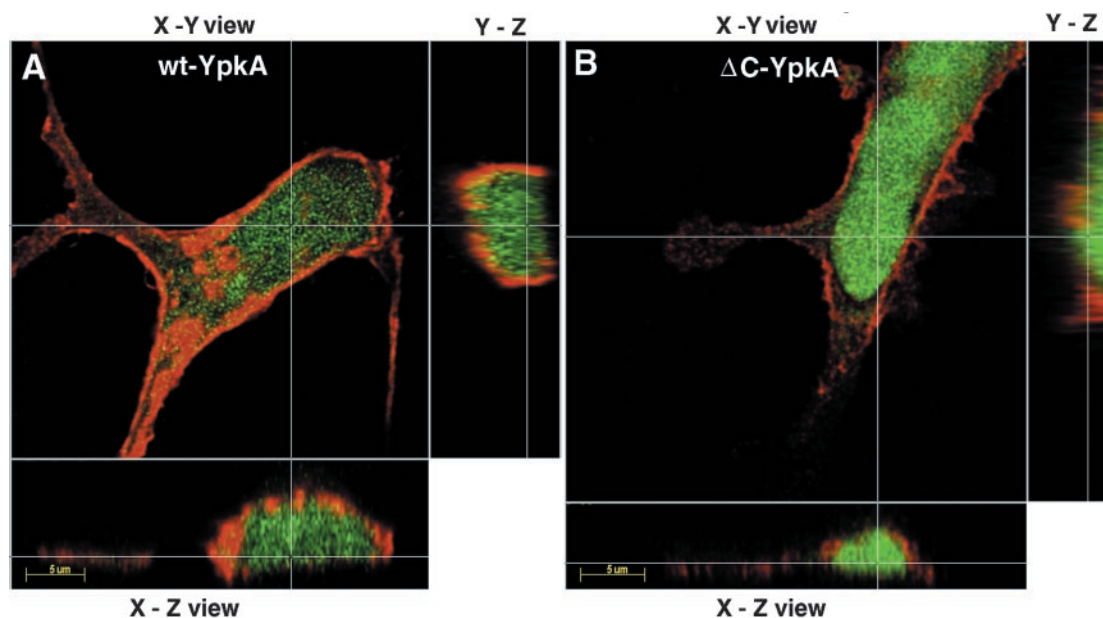


FIG. 3. Intracellular localization of YpkA in transfected cells. Twenty hours after transfection GFP expression was detected directly (green) while YpkA expression was analyzed by immunostaining (red). The images show a cut through the point in the x , y , and z axes (x - y , y - z , and x - z view). 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.

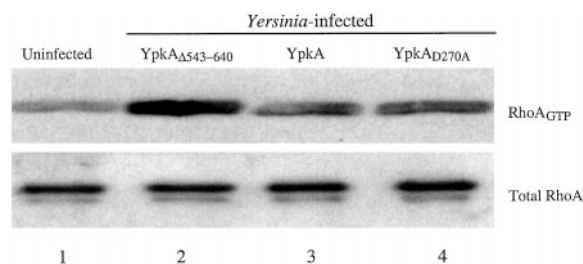


FIG. 4. Recovery of active RhoA from either uninfected or *Yersinia*-infected cells. Serum-starved HeLa cells were either left uninfected or infected with *Yersinia* expressing the indicated variants of YpkA for 80 min. Whole cell lysates were mixed with GST-rhotekin_{RBD}-bound beads and the levels 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 [3 H]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-Lsc, 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.

Chromatography—A HeLa cytosolic extract was prepared by removing attached cells grown to confluence from two 175-cm² with trypsin, resuspending the cells in 2 ml of PBS, 0.25 M sucrose, and sonicating the cells for 10 s in 0.5-ml volumes. Sonicates were spun first at low-speed in a microcentrifuge and then at 100,000 × g for 1 h at 4 °C. The resulting supernatant was applied to a Sephacryl S-300-containing column with a total volume of 340 ml and eluted with PBS at a flow-rate of 20 ml/h. A prefraction was collected followed by 100 fractions of 2.5 ml each. Fractions were assayed for their kinase promoting activity by combining 1 ml of each fraction with bead-bound GST-RhoA/YpkA (described above), incubating for 30 min at 4 °C, and resuspending the beads in kinase buffer. The kinase assay was performed as described above. For serum fractionation, 4 ml of fetal calf serum was applied to the same S-300 column used for the HeLa extract and eluted using

essentially the same conditions. Serum fractions were analyzed by adding 20 μ l of each fraction directly to the kinase assay described above. The column was calibrated under the same conditions with blue dextran, human IgG, human serum albumin, ovalbumin, and tritiated water.

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 (YpkA $_{\Delta 543-640}$), 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 YpkA $_{\Delta 543-640}$ 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 $\Delta 543-640$ 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

	Helix 2	Helix 3
PKN(ACC-1)	I R K E L K L K E G A E N L R R...17...R G S S R R L D L L H	
YpkA ₄₃₉	R I T P K K L R E L S D L L R T...31...R E G G V D K D Q L K	
YpkA ₅₀₈	I E D Y V K G R E G D T K N S S...18...Q R I Q K H L D Q T H	
YpkA ₅₉₉	A E A K I T L S Q Q L N T L Q Q...23...R Q S L Q R F D S T R	
YpkA ₇₀₈	V E Q R E K L R E L T T I A E R (24)	Q E V S E F T D D M R
hum p160 _{ROCK}	E E L T E K M K K A E E Y K L...26...Q A V N K L A E I M N	
bov Rho-kinase	E E L N N K L K E A Q E Q L S R...28...Q A V N K L A E I M N	
hum rhotekin	L D I E L K V K Q G A E N M I Q...22...Q D N K T K I E V I R	
mus kinectin	K R I E V K L K D T E S D V S K (79)	Q A L K Q E I E V L K
PKN(ACC-2)	L A I E L K V K Q G A E N M I Q...20...Q D S K T K I D I I R	

FIG. 5. Sequence alignment of the RhoA interaction domain of PKN/PRK1 with YpkA and other RhoA-binding kinases. PKN/PRK1 residues which make direct contacts with RhoA, as determined by x-ray crystallography (28), are displayed in outline as well as similar residues found at the corresponding positions in YpkA and the other kinases. The PKN/PRK1 RhoA interaction domain consist of two helices (2 and 3) that are connected by a loop of four residues. Shown *above* is the number of residues that separate the end of the RhoA interaction subdomain within helix 2 and the beginning of the RhoA interaction subdomain within helix 3. In the fourth YpkA repeat (YpkA₇₀₈) and mouse kinectin the predicted RhoA-binding domain "helix 3" precedes "helix 2" in the primary sequence (in *parentheses* is shown the number of residues which separate the end of the predicted RhoA interaction subdomain within helix 3 and the beginning of the predicted RhoA interaction subdomain within helix 2). Amino acid residues were grouped as either hydrophobic (Leu, Ile, Val, Ala, Phe, Met, Gly, and Pro), polar (Asn, Gln, Ser, Thr, Tyr, Cys, and Trp), acidic (Asp and Glu), or basic (Arg, Lys, and His).

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_{GTP} 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_{GTP} in *Yersinia*-infected cells. Using a RhoA_{GTP}-specific affinity purification assay (24) we observed a slight increase in the amount of RhoA_{GTP} recovered in cells infected with *Yersinia* expressing either the wild type or D270A variants of YpkA compared with the amount of RhoA_{GTP} recovered in uninfected cells (Fig. 4). However, significantly more RhoA_{GTP} was recovered in cells infected with *Yersinia* express-

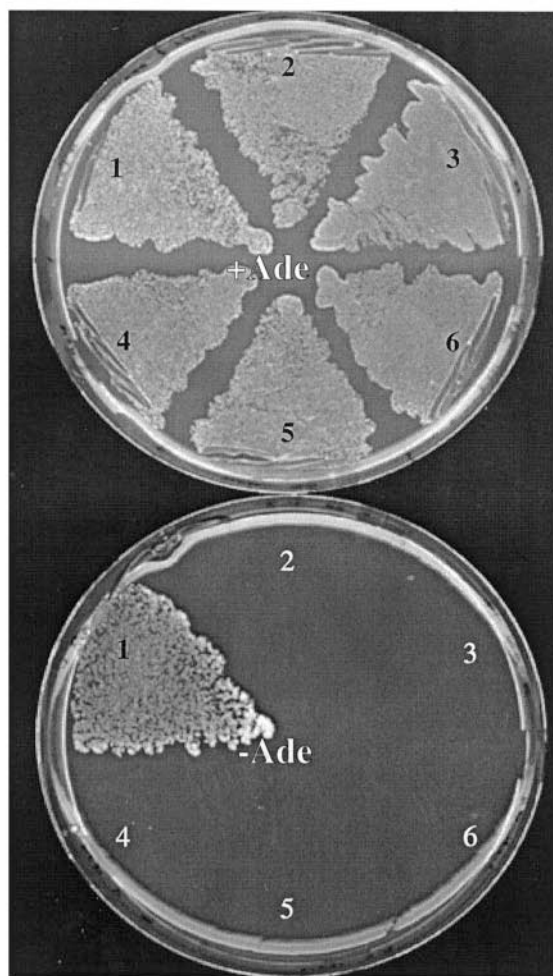
ing the $\Delta 543$ –640 variant of YpkA compared with either the uninfected cells or the cells infected with *Yersinia* expressing full-length YpkA variants (compare lanes 2 with 1, 3, and 4). These data indicate that under these infection and assay conditions the carboxyl-terminal domain of YpkA affects the levels of active RhoA_{GTP} 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_{BD}-YpkA₄₄₂₋₇₃₃ hybrid protein, containing all four pairs of the predicted RhoA-interacting helices, tested positive for interaction with a Gal4_{AD}-RhoA hybrid protein in a yeast two-hybrid assay (Fig. 6A). In contrast, yeast containing plasmids encoding Gal4_{AD}-RhoA and either Gal4_{BD}-YpkA₄₄₂₋₇₃₃($\Delta 543$ –640) or Gal4_{BD}-YpkA₉₇₋₃₄₇ did not express the reporter gene (Fig. 6A). (The $\Delta 543$ –640 YpkA variant is missing three helices in the middle of YpkAs RBD.) To determine whether the Gal4_{BD}-YpkA₄₄₂₋₇₃₃/Gal4_{AD}-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_{BD}-YpkA₄₄₂₋₇₃₃, Gal4_{AD}-RhoA, and either YpkA₄₄₉₋₇₃₃ or RhoA. Transformants expressing either of the competitors (*i.e.* YpkA₄₄₉₋₇₃₃ 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_{BD}-YpkA₄₄₂₋₇₃₃ and Gal4_{AD}-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 _{$\Delta 543$ –640}) 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.

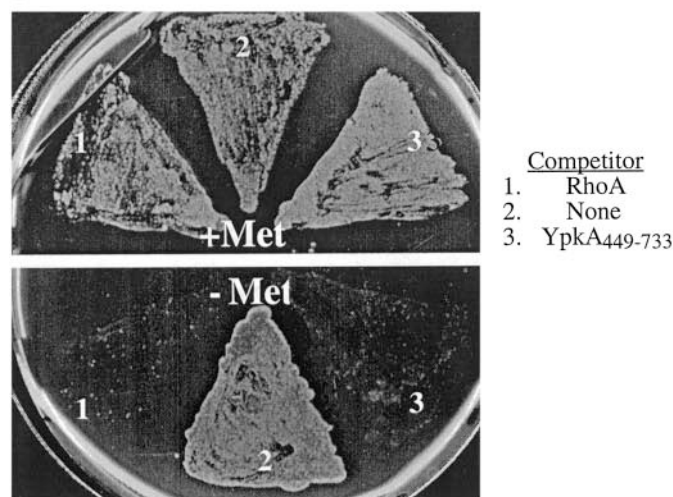
Nearly all the eukaryotic RhoA-binding kinases described to

A



1. pGAL4_{BD}-YpkA₄₄₂₋₇₃₃/pGAL4_{AD}-RhoA
2. pGAL4_{BD}-YpkA₄₄₂₋₇₃₃(Δ 543-640)/pGAL4_{AD}-RhoA
3. pGAL4_{BD}-YpkA₉₇₋₃₄₇/pGAL4_{AD}-RhoA
4. pGAL4_{BD}-YpkA₄₄₂₋₇₃₃/pGAL4_{AD}
5. pGAL4_{BD}-YpkA₄₄₂₋₇₃₃(Δ 543-640)/pGAL4_{AD}
6. pGAL4_{BD}-YpkA₉₇₋₃₄₇/pGAL4_{AD}

B



- Competitor
1. RhoA
 2. None
 3. YpkA₄₄₉₋₇₃₃

FIG. 6. Yeast two-hybrid assays. A, yeast were co-transformed with various combinations of bait plasmids containing different regions of YpkA and either Gal4_{AD}-RhoA- (1–3) or Gal4_{AD}-encoding vectors

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 *in vitro* binding assays. To test whether YpkA displays similar binding characteristics, yeast were co-transformed with plasmids encoding Gal4_{BD}-YpkA₄₄₂₋₇₃₃ and Gal4_{AD}-V14-RhoA (the constitutively active form of RhoA). No growth of the Gal4_{BD}-YpkA₄₃₈₋₇₃₃/Gal4_{AD}-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, p160_{I_{rock}} (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 γ 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_{GDP} compared with the amount of YpkA that copurified with either GST-RhoA_{GTP} or GST-RhoA_{GTP- γ S} (Fig. 7C, upper panel). Preferential YpkA binding to GDP-bound RhoA was observed over an 80-fold range of input YpkA (Fig. 7D). Approximately equal levels of YpkA binding was observed in assays using “unloaded” GST-RhoA (as used in Fig. 7A, see above) and GST-RhoA loaded with GDP (not shown). In our GST pull-down assays, YpkA appeared to have approximately equal affinities for the GDP- and GTP-bound forms of Rac (see Fig. 9C).

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 Δ 543–640 (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 Gal4_{AD}-RhoA-encoding plasmids and Gal4_{BD}-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).

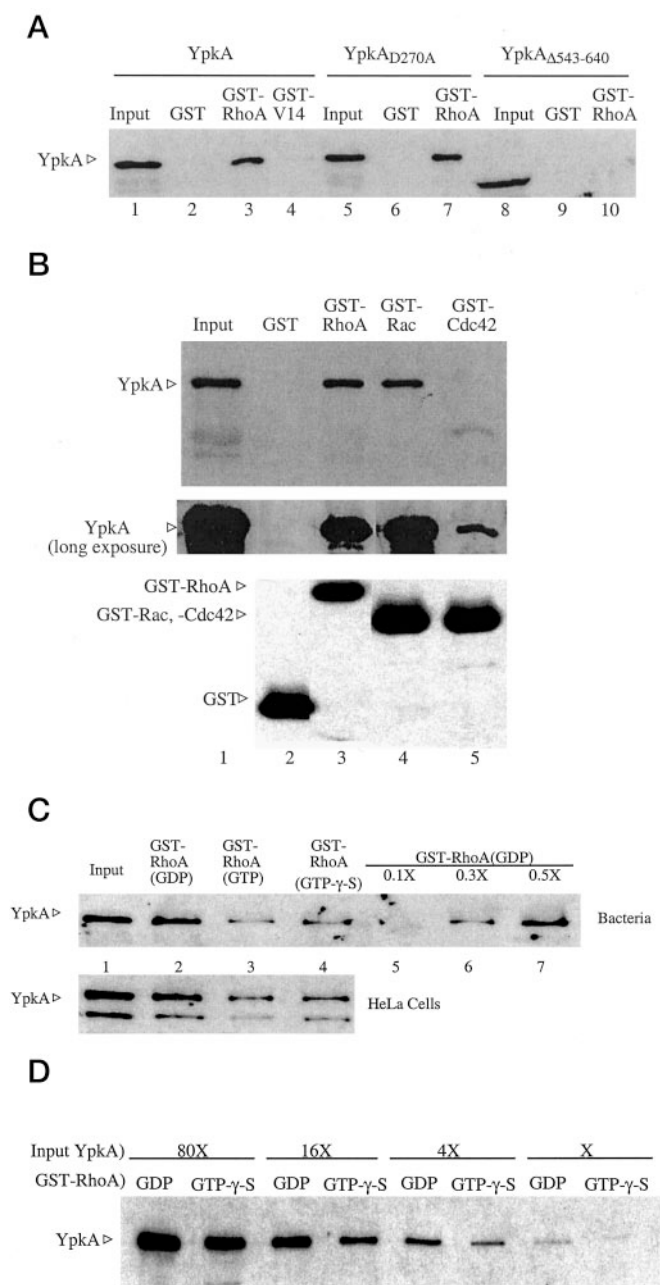


FIG. 7. GST pull-down assays. A, cytoplasmic extracts prepared from *Yersinia* strains expressing wild type YpkA (lane 1), a YpkA kinase point mutant (lane 5), or YpkA with an internal deletion of residues 543–640 (lane 8), were mixed with glutathione beads bound with GST (lanes 2, 6, and 9), GST-RhoA (lanes 3, 7, and 10), or GST-Val¹⁴-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 GTP- γ S (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 5) were run in lanes 5–7 for comparative purposes. D, a constant amount of either bead-bound GST-RhoA^{GDP} or GST-RhoA^{GTP- γ S} were mixed with various amounts of *Yersinia*-expressed YpkA and processed and analyzed as in A.

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-

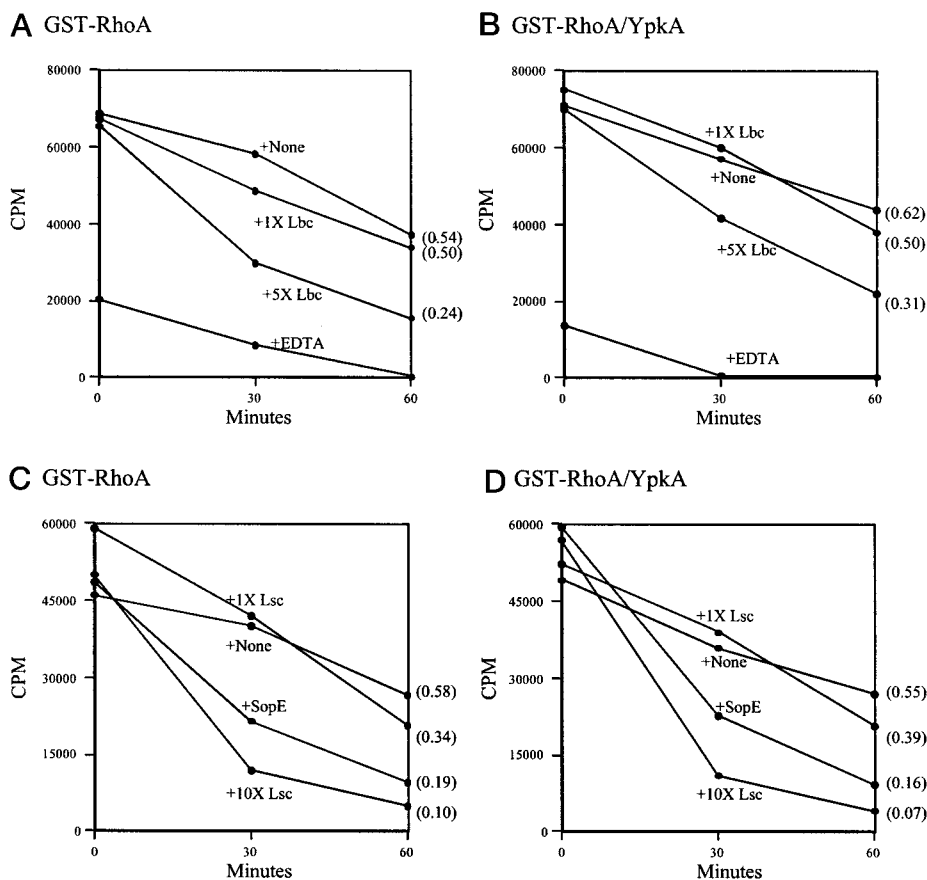


FIG. 8. **GDP dissociation assays.** Either unbound (A and C) or YpkA-bound (B and D) GST-RhoA[8-³H]GDP-loaded glutathione beads (approximately 1 μ g of protein) were incubated at 30 °C with the indicated supplements. At the indicated time points aliquots were removed and the aliquoted beads were washed and resuspended in scintillation fluid and counted for 1 min. The amount of supplements added were: GST-Lbc and GST-Lsc, X = 100 ng; GST-SopE, 800 ng; EDTA, 10 mM. 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.

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 YpkAs kinase activity when bound to either GDP- or GTP γ S-bound RhoA or Rac. There were no apparent differences in kinase activity observed between RhoA_{GDP}/Rac_{GDP}-bound YpkA and RhoA_{GTP γ S}/Rac_{GTP γ S}-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 YpkAs 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 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 interac-

tion 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 kinase family as well as IKK β (33). The targeting of mitogen-activated protein kinases/IKK β 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

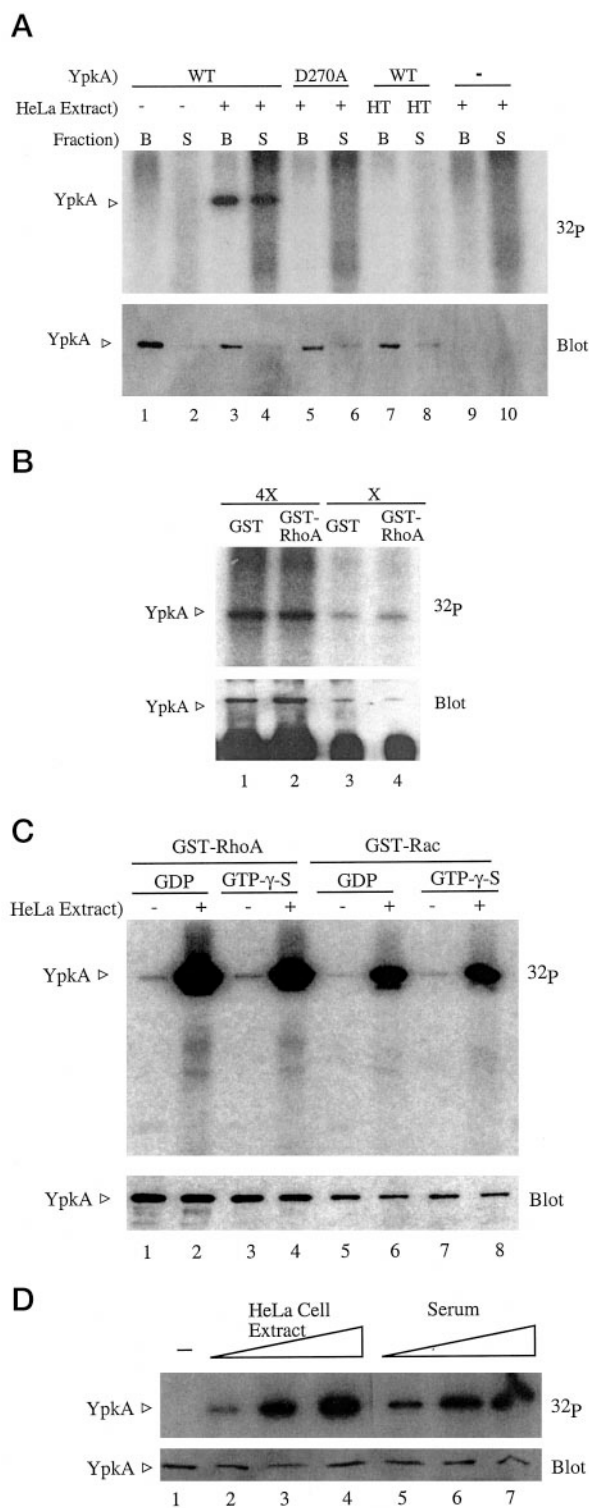


FIG. 9. YpkA kinase activity. A, GST-RhoA-loaded beads were mixed with cytoplasmic extracts prepared from *Yersinia* strains expressing wild type YpkA (lanes 1–4, 7, and 8), the D270A point mutant (lanes 5 and 6), or no YpkA (lanes 9 and 10). Following the binding reaction beads were resuspended in $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -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 ^{32}P -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-

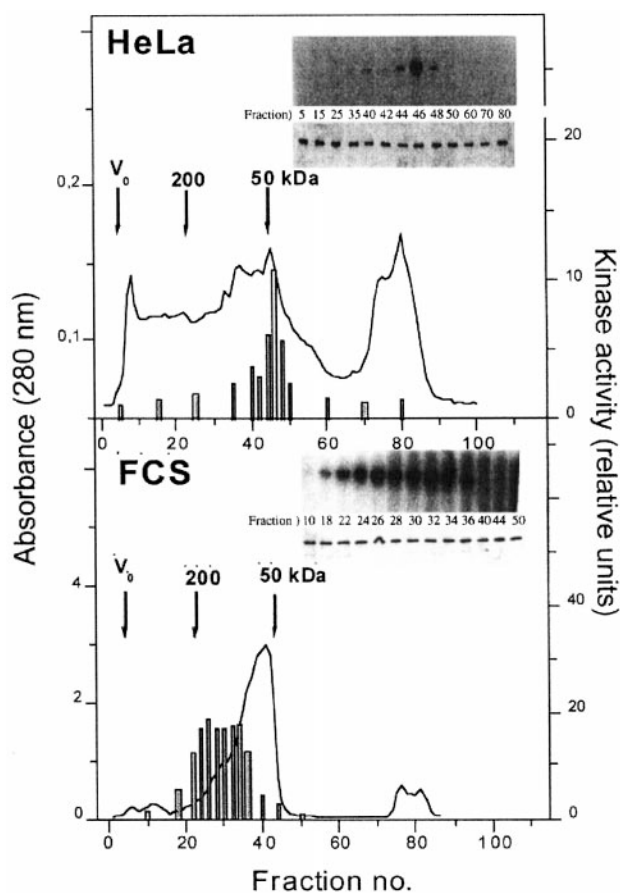


FIG. 10. Chromatographic properties of the HeLa- and serum-derived YpkA-inducing factors. Either a 100,000 $\times g$ cytosolic HeLa cell extract or untreated fetal calf serum were fractionated using Sephacryl S-300. Fractions were measured for protein content (left Y axis) and used in a YpkA kinase assay (inset, ^{32}P 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 $^{\circ}\text{C}$, and then collected and resuspended in $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ -containing kinase buffer and analyzed as in Fig. 7A. Serum fractions were added directly to the kinase reaction as in Fig. 7A. The ^{32}P signal was quantified by a luminescent image analyzer (Fujifilm) and reported relative to the lowest signal (bars, right y axis).

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/HR1b^{PRK1} 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/HR1a^{PRK1}, 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

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.

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 YpkAs carboxyl-terminal located RhoA/Rac-binding domain related to the amino-terminal located kinase domain? In this study we show that YpkAs RhoA binding and enzymatic activities appear to be independent of one another. YpkAs 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 YpkAs amino terminus. Thus it remains to be determined the relationship, if any, between YpkAs 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 different. 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 modifying toxin (ExoS), respectively (42–44). In these latter examples it is thought that the host cell activator is simply an abundant cytosolic protein that serves as an “on” switch for the toxin. Therefore identifying the YpkA host cell activators may not necessarily reveal much about the biological activity of YpkA. However, the fact that YpkA responds to a serum factor is intriguing and may indicate that YpkA possesses an extracellular function in addition to its predicted intracellular activity.

YpkA is not the only injected *Yersinia* protein affecting the Rho GTPases. Recently, YopE, has been demonstrated to possess GTPase activating protein activity toward the Rho GTPases and that this activity is required for the actin disrupting activity of YopE (23, 45, 46). Since YopE is expressed and injected at levels far exceeding that of YpkA, it is likely that the majority of Rho proteins in a *Yersinia*-infected cell are GDP bound. Additionally, the activity of YopH, a protein-tyrosine phosphatase that targets focal adhesion kinase and disrupts integrin-mediated signaling (47, 48), could also affect the activation status of the Rho proteins (49). And finally, *Yersinia enterocolitica*, but not all strains of *Yersinia pestis* and *Yersinia pseudotuberculosis*, expresses an additional virulence protein, YopT, which is involved in disruption of the actin cytoskeleton (50). This actin disrupting activity of YopT likely involves RhoA since in eukaryotic cells infected with YopT-expressing *Y. enterocolitica* RhoA is covalently modified (51). Thus precisely whether and how YpkA affects RhoA activity *in vivo* remains to be determined and must be considered in the context of the activities of the other Yop virulence proteins. Until the substrates of YpkA are determined the roles played by its inducible kinase and RhoA binding activities in the infection process will remain speculative.

Acknowledgments—We thank Tomas Leanderson, Eva Miller, Scott Kuersten, Brahim Houssa, Ulrich von Pawel-Rammingen, Lena Wester, Axel Åkerström, Edouard Galyov, Lawrence A. Quilliam, Judy

Glaven, Paschalis Sideras, Karim Dib, Fredrik Meander, Xiao-Dong Su, and Eva Degerman for their generosity and helpfulness.

REFERENCES

1. Salmond, G. P., and Reeves, P. J. (1993) *Trends Biochem. Sci.* **18**, 7–12
2. Cornelis, G. R., and Wolf-Watz, H. (1997) *Mol. Microbiol.* **23**, 861–867
3. Viprey, V., Del Greco, A., Golinowski, W., Broughton, W. J., and Perret, X. (1998) *Mol. Microbiol.* **28**, 1381–1389
4. Yang, Y., Shah, J., and Klessig, D. F. (1997) *Genes Dev.* **11**, 1621–1639
5. Galán, J. E., and Collmer, A. (1999) *Science* **284**, 1322–1328
6. Rosqvist, R., Bölin, I., and Wolf-Watz, H. (1988) *Infect. Immun.* **56**, 2139–2143
7. Nakajima, R., and Brubaker, R. (1993) *Infect. Immun.* **61**, 23–31
8. Beuscher, H., Rödel, F., Forsberg, Å., and Rölinghoff, M. (1995) *Infect. Immun.* **63**, 1270–1277
9. Schulte, R., Wattiau, P., Hartland, E., Robin-Browne, R., and Cornelis, G. R. (1996) *Infect. Immun.* **64**, 2106–2113
10. Galyov, E. E., Håkansson, S., Forsberg, Å., and Wolf-Watz, H. (1993) *Nature* **361**, 730–731
11. Galyov, E. E., Håkansson, S., and Wolf-Watz, H. (1994) *J. Bacteriol.* **176**, 4543–4548
12. Håkansson, S., Galyov, E. E., Rosqvist, R., and Wolf-Watz, H. (1996) *Mol. Microbiol.* **20**, 593–603
13. Van Aelst, L., and D'Souza-Schorey, C. (1997) *Genes Dev.* **11**, 2295–2322
14. Mackay, D. J. G., and Hall, A. (1998) *J. Biol. Chem.* **273**, 20685–20688
15. Schmidt, G., Sehr, P., Wilm, M., Selzer, J., Mann, M., and Aktories, K. (1997) *Nature* **387**, 725–729
16. Flatau, G., Lemichez, E., Gauthier, M., Chardin, P., Paris, S., Fiorentini, C., and Boquet, P. (1997) *Nature* **387**, 729–730
17. Horiguchi, Y., Inoue, N., Masuda, M., Kashimoto, T., Katahira, J., Sugimoto, N., and Matsuda, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11623–11626
18. Aktories, K., Braun, U., Rosener, S., Just, I., and Hall, A. (1989) *Biochem. Cell Biol.* **158**, 209–213
19. Wood, M. W., Rosqvist, R., Mullan, P. B., Edwards, M. H., and Galyov, E. E. (1996) *Mol. Microbiol.* **22**, 327–338
20. Hardt, W.-D., Chen, L.-M., Schuebel, K. E., Bustelo, X. R., and Galán, J. E. (1998) *Cell* **93**, 815–826
21. Fu, Y., and Galán, J. E. (1999) *Nature* **401**, 293–297
22. James, P., Halladay, J., and Craig, E. A. (1996) *Genetics* **144**, 1425–1436
23. Rosqvist, R., Forsberg, Å., and Wolf-Watz, H. (1991) *Infect. Immun.* **59**, 4562–4569
24. Ren, X.-D., Kioussis, W. B., and Schwartz, M. A. (1999) *EMBO J.* **18**, 578–585
25. Vincent, S., and Settleman, J. (1997) *Mol. Cell Biol.* **17**, 2247–2256
26. Bölin, I., and Wolf-Watz, H. (1984) *Infect. Immun.* **43**, 72–78
27. Glaven, J. A., Whitehead, I. P., Nomanbhoy, T., Kay, R., and Cerione, R. A. (1996) *J. Biol. Chem.* **271**, 27374–27381
28. Maesaki, R., Ihara, K., Shimizu, T., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1999) *Mol. Cell* **4**, 793–803
29. Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J., and Egly, J. M. (1997) *J. Biol. Chem.* **272**, 22995–22999
30. Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996) *EMBO J.* **15**, 1885–1893
31. Lang, P., Gesbert, F., Delespine-Carmagnat, M., Stancou, R., Pouchelet, M., and Bertoglio, J. (1996) *EMBO J.* **15**, 510–519
32. Houssa, B., de Widt, J., Kranenburg, O., Moolenaar, W. H., and van Blitterswijk, W. J. (1999) *J. Biol. Chem.* **274**, 6820–6822
33. Orth, K., Palmer, L. E., Qin Bao, Z., Stewart, S., Rudolph, A. E., Bliska, J. B., and Dixon, J. E. (1999) *Science* **285**, 1920–1923
34. Boland, A., and Cornelis, G. R. (1998) *Infect. Immun.* **66**, 1878–1884
35. Palmer, L. E., Hobbie, S., Galán, J. E., and Bliska, J. B. (1998) *Mol. Microbiol.* **27**, 953–965
36. Schesser, K., Spiik, A.-K., Dukuzumuremyi, J.-M., Neurath, M. F., Pettersson, S., and Wolf-Watz, H. (1998) *Mol. Microbiol.* **28**, 1067–1079
37. Flynn, P., Mellor, H., Palmer, R., Panayotou, G., and Parker, P. J. (1998) *J. Biol. Chem.* **273**, 2698–2705
38. Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamjima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) *Science* **271**, 648–650
39. Leung, T., Manser, E., Tan, L., and Lim, L. (1995) *J. Biol. Chem.* **270**, 29051–29054
40. Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) *Science* **273**, 245–248
41. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996) *J. Biol. Chem.* **271**, 20246–20249
42. Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullman, A., and Danchin, A. (1988) *Mol. Microbiol.* **2**, 19–30
43. Fu, H., Coburn, J., and Collier, R. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2320–2324
44. Henriksson, M., Troller, U., and Hallberg, B. (2000) *Biochem. J.* **349**, 697–701
45. Von Pawel-Rammingen, U., Telepnev, M. V., Schmidt, G., Aktories, K., Wolf-Watz, H., and Rosqvist, R. (2000) *Mol. Microbiol.* **36**, 737–748
46. Black, D. S., and Bliska, J. B. (2000) *Mol. Microbiol.* **37**, 515–527
47. Persson, C., Carballeira, N., Wolf-Watz, H., and Fällman, M. (1997) *EMBO J.* **16**, 2307–2318
48. Black, D. S., and Bliska, J. B. (1997) *EMBO J.* **16**, 2730–2744
49. Clark, E. A., King, W. G., Brugge, J. S., Symons, M., and Hynes, R. O. (1998) *J. Cell Biol.* **142**, 573–586
50. Iriarte, M., and Cornelis, G. R. (1998) *Mol. Microbiol.* **29**, 915–929
51. Zumbihl, R., Aepfelbacher, M., Andor, A., Jacobi, C. A., Ruckdeschel, K., Rouot, B., and Heesemann, J. (1999) *J. Biol. Chem.* **274**, 29289–29293