The *Yersinia* Ser/Thr protein kinase YpkA/YopO directly interacts with the small GTPases RhoA and Rac-1

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Abstract Pathogenic bacteria of the genus *Yersinia* counteract host defense by interfering with eukaryotic signal transduction pathways. YpkA of *Yersinia pseudotuberculosis* shares significant homology with eukaryotic Ser/Thr protein kinases, is translocated into the host cell and has been shown to be an essential virulence factor in a mouse infection model. In this study, we identify the small GTPases RhoA and Rac-1 as eukaryotic binding partners of YpkA and its homolog YopO of *Yersinia enterocolitica*. We demonstrate that the interaction is independent of phosphorylation of YpkA and nucleotide loading state of the GTPases. The interaction with RhoA and Rac-1 might provide an important clue to how YpkA interferes with eukaryotic signaling on a molecular level.

Key words: Rho; Rac; Yop; YopO; YpkA; *Yersinia*

1. Introduction

The enteropathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* cause food-borne diseases, ranging from mild self-limited diarrhea to mesenteric adenitis. A major clue to *Yersinia* pathogenicity is the presence of a 70 kb virulence plasmid (pYV) which is highly conserved among the two enteropathogenic *Yersinia* species and *Yersinia pestis*, the causative agent of plague. The pYV plasmid consists of two functional divergent regions: one encodes the protein type III secretion/translocation apparatus and transcriptional regulators whereas the other region encodes several effector proteins (*Yersinia* outer proteins, Yops) [1]. The type III secretion system enables the bacteria to translocate these effector proteins into the host cell thus interfering with host signal transduction and other cellular functions. Up to now, six Yops of *Y. enterocolitica*, termed YopE, YopH, YopM, YopO (YpkA in *Y. pseudotuberculosis*), YopP (YopJ in *Y. pseudotuberculosis*) and Yop T, have been described [2–4]. All of them have been detected in the cytosol of infected cells and, except YopM, have been shown to alter cellular functions to various extents [5–14].

ypkA was cloned from *Y. pseudotuberculosis* and characterized first by Galyov et al. [15,16]. Because of a noticeable homology to eukaryotic Ser/Thr protein kinases, the protein was called '*Yersinia* protein kinase A' (YpkA) [15]. In vitro kinase assays revealed that YpkA possesses autophosphorylating activity [15]. Infection of BALB/c mice with *Y. pseudotuberculosis* strains defective in *ypkA* expression gave evidence that YpkA is essential for virulence since all animals survived infection with this *Yersinia* strain whereas challenge with identical numbers of wild-type *Y. pseudotuberculosis* was lethal in all cases [15].

Using a YpkA overexpressing strain of *Y. pseudotuberculosis* in a HeLa cell infection model, it was shown that YpkA was translocated and targeted to the inner surface of the HeLa cell membrane. Infection altered HeLa cell morphology in a way different from the cytotoxic changes attributed to YopE and was characterized primarily by pronounced and branched retraction fibers [17]. However, the target protein linking YpkA to the observed effects has not been identified yet.

Here, we report that two small GTPases of the Rho family, Rac-1 and RhoA, directly interact with YpkA in a yeast two-hybrid assay, in vitro using recombinant proteins and immunoprecipitation. This interaction is independent of phosphorylation of YpkA and the activation state of the small GTPase characterized by their binding to GDP or GTP.

2. Materials and methods

2.1. Yeast two-hybrid system

The DupLEX-A Yeast Two-Hybrid System (OriGene Technologies, Inc., Rockville, MD, USA) was used to screen for potential binding partners of YopO following the instructions of the manufacturer. Briefly, the gene encoding YopO was amplified from *Y. enterocolitica* WA-314 (Table 1) (forward primer: ATATAGAATTCATGAAAATCATGGGAACTATGTC; reverse primer: ATATAGTCGACTCACATCCATTCCCGCTCCA) and cloned into pEG202 using *Eco*RI and *Sal*I restriction sites, resulting in pEG202-*yopO*.

The fusion of *yopO* and *lexA* was expressed in *Saccharomyces cerevisiae* EGY48 and was shown not to activate the expression of the *lacZ* and *LEU2* reporters. EGY48 harboring pEG202-*yopO* was then transformed with a HeLa cell library (OriGene Technologies, Inc., Rockville, MD, USA). Transformed yeast clones were first selected on minimal media plates lacking leucine (−Leu plates). The resulting Leu^+ colonies were then screened for *lacZ* expression in filter assays. Positive yeast clones were further investigated by recovering library plasmids from these clones and retransforming them into *S. cerevisiae* strains harboring pEG202-*yopO* or various control plasmids, respectively. Library plasmids that tested again positive for induction of *LEU2* and *lacZ* expression in EGY48 harboring pEG202-*yopO* but did not induce expression in EGY48 harboring negative control plasmids were sequenced. The gene encoding YpkA was amplified from *Y. pseudotuberculosis* YPIII (pIB102) (Table 1) (forward primer: ATATACCATGGATGAAAAGCGTGAAAATCATGGG; reverse primer: ATATACTCGAGTCACATCCATTCCCGCTCCA) and cloned into pEG202 using *Nco*I and *Xho*I as restriction sites, resulting in pEG202-*ypkA*.

2.2. Expression and purification of recombinant YpkA

Recombinant YpkA was expressed in *Escherichia coli* DH5α using the IMPACT I protein purification system (New England Biolabs GmbH, Schwalbach, Germany) according to the instructions of the

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manufacturer. The gene encoding YpkA was amplified from *Y. pseudotuberculosis* YPIII (pIB102) (forward primer: ATATACATATGATGAAAATCATGGGAACTATGTCAC; reverse primer: ATATACCCGGGTCACATCCATTCCCGCTCCAACCG) and cloned into pTYB2 (New England Biolabs GmbH, Schwalbach, Germany) using *Nde*I and *Sma*I as restriction sites.

2.3. Expression of recombinant GST-RhoA, GST-Rac-1 and GST-Cdc42

Recombinant RhoA, Rac-1 and Cdc42 were expressed as GST fusion proteins. Plasmids encoding the small GTPase fusion constructs were a kind gift from Dr. Alan Hall (University College, London, UK) and were prepared as described previously [18].

2.4. Preparation of antibodies specific to YpkA

Polyclonal rabbit antiserum was raised against recombinant YpkA expressed and purified as described above. Two female New Zealand white rabbits were immunized with recombinant YpkA. Antisera were obtained on day 60 and used without purification for immunoprecipitation and Western blot analysis.

2.5. Growth and preparation of bacterial strains for infection of host cells

Yersinia cultures were grown in LB broth at 27°C overnight, diluted 1:20 with fresh LB broth and cultivated for 2 h at 37°C. Bacteria were washed once and resuspended in phosphate-buffered saline (PBS). Bacteria were used to infect host cells at the indicated multiplicity of infection. The desired bacterial concentration was adjusted by measuring the optical density at 600 nm.

COS cells and HeLa cells were cultured in Dulbecco's modified Eagle medium (Life Technologies, Cergy, Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum and 5 mM L-glutamine. Infection of cells was performed at 37°C for time periods indicated.

2.6. Coimmunoprecipitation and Western blotting

6×10^7 COS cells were infected with WA-C (pTRANS, pCJYE138-*ypkA*) (Table 1) for 2 h at 37°C at a ratio of 50 bacteria/host cell. Cells were washed twice with ice-cold PBS, scraped off and pelleted by centrifugation. Cells were lysed in 300 μl ice-cold lysis buffer (20 mM Tris, pH 7.5, 3 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, 1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin) for 1 h at 4°C. The lysate was cleared by centrifugation. 50 μl of supernatant was directly added to sodium dodecyl sulfate (SDS) sample buffer and used as a marker for the total amount of protein present. 250 μl of supernatant was combined with 50 μl protein G-agarose beads (Boehringer Mannheim, Germany) and 6 μl polyclonal anti-YpkA rabbit serum. Immunoprecipitation was performed overnight at 4°C. Beads were washed three times with lysis buffer, resuspended in sample buffer, boiled and proteins were separated by 15% SDS–PAGE. After Western blotting, the membrane was blocked and probed for RhoA, Rac-1 or Cdc42, respectively. Anti-RhoA antibodies were purchased from Santa Cruz Biotechnologies, USA. Anti-Rac-1 and anti-Cdc42 antibodies were purchased from Transduction Laboratories, San Diego, CA, USA.

2.7. Assay for YpkA phosphorylation

Recombinant YpkA was bound to anti-YpkA rabbit serum and immobilized on protein G-agarose beads (Boehringer Mannheim, Germany) for 1 h at 4°C. Beads were washed three times in kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM dithiothreitol (DTT), 20 mM ATP). Samples were subjected to kinase reaction with 1 μCi [γ-^{32}P]ATP per sample for 30 min at 30°C directly or after incubation with 2 μl of HeLa cell lysate (cleared lysate of approximately 10^5 HeLa cells) for 5 min at room temperature. Samples were washed in kinase buffer, resuspended in sample buffer, boiled and separated by SDS–PAGE. The dried gel was subjected to autoradiography.

2.8. In vitro binding studies of YpkA to RhoA, Rac-1 and Cdc42

GST-RhoA, GST-Rac-1 and GST-Cdc42 were bound to glutathione-Sepharose beads (Pharmacia, Uppsala, Sweden) for 1 h at 4°C. The beads were washed three times with nucleotide depletion buffer (20 mM Tris pH 7.5, 1 mM DTT, 10 mM EDTA, 50 mM NaCl, 5% glycerol, 0.1% Triton X-100) and incubated for 20 min at room temperature to deplete the GTPases of GDP and GTP. To load the fusion proteins with GDP or GTP, aliquots where washed three times with GDP loading buffer (20 mM Tris, 1 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 0.1% Triton X-100, 200 μM GDP) or GTP loading buffer (20 mM Tris, 1 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 5% glycerol, 0.1% Triton X-100, 20 μM GTP (5′ guanylylimidodiphosphate, Sigma)) and incubated for 25 min at room temperature. Recombinant YpkA with or without HeLa cell lysate (cleared lysate of approximately 10^5 HeLa cells) was added to kinase buffer (50 mM HEPES pH 7,5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM DTT, 20 mM ATP) and incubated at 30°C for 30 min. After kinase reaction, YpkA was combined with the GST-GTPases loaded with GTP, GDP or without any nucleotide. Samples were tumbled on a rotating wheel for 1 h at 4°C. Beads were washed twice with nucleotide depletion buffer, GDP loading buffer or GTP loading buffer, respectively. Samples were boiled and separated on SDS–PAGE. Proteins were visualized using Coomassie stain. To test for activity of the GST-RhoA fusion, GST-RhoA was loaded with either GTPγ-S or GDPβ-S and investigated for binding to the Rho-binding domain of the effector Rhotekin (GST-C21). The GST-RhoA protein in the GTPγ-S but not in the GDPβ-S-bound form was able to pull down the Rho-binding domain of Rhotekin.

3. Results

3.1. YopO and YpkA interact with Rac-1 and RhoA but not with Cdc42 in the yeast two-hybrid system

Full length *yopO* was amplified from *Y. enterocolitica* WA-314 (Table 1) by PCR, cloned in frame into a yeast *lexA* expression vector (pEG202) and transformed into yeast strain EGY48. Approximately 1×10^7 clones of a HeLa cell expression library were screened for interaction with YopO. Sequencing of the DNA inserts of 32 library plasmids recovered from the screen revealed 15 gene fragments that were not further investigated in this study and 17 inserts encoding Rac-1, a member of the Rho family of small GTPases. Of these 17 Rac-1 inserts, four were independent clones having different 5′ and 3′ termini, pointing to a true positive in the

Table 1
Plasmids and *Yersinia* strains used in this study

Strain or plasmid	Characteristics	Ref.
pCJYE138-*ypkA*	pACYC184 vector plasmid carrying a *Sal*I–*Hin*dIII fragment consisting of sycE encoding YopE chaperone and truncated *yopE* gene with 138 codons [29] translationally fused in frame to full length *ypkA*	this study
WA-314	*Y. enterocolitica* serogroup O8, clinical isolate harboring virulence plasmid pYVO8	[30]
YPIII (pIB102)	*Y. pseudotuberculosis*, parental strain	[15]
WA-C (pTRANS)	*Y. enterocolitica*, serotype O8, carrying plasmid pTRANS encoding the type III secretion/translocation region and YadA of pYVO8. pTRANS was obtained from pLCR by replacement of the vector plasmid pSUP102 through pLAF as described previously [31]	this study
WA-C (pTRANS, pCJYE138-*ypkA*)	WA-C (pTRANS), complemented with pLAF-lcr encoding the type III secretion system with YadA and pCJYE138-*ypkA*	this study

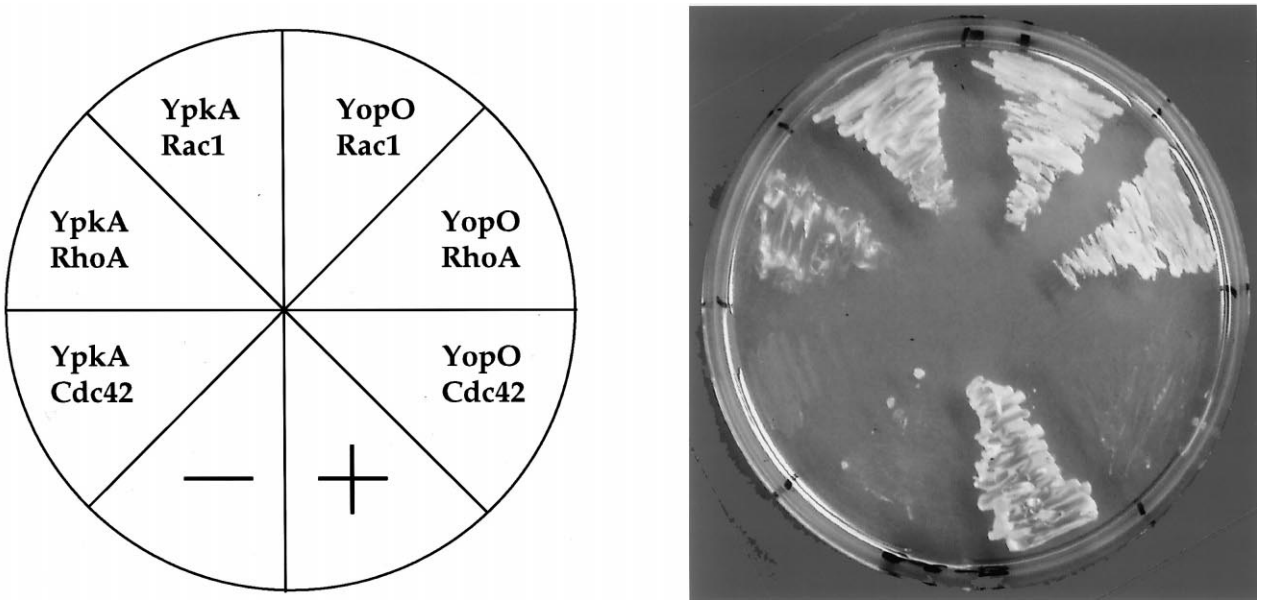

Fig. 1. YpkA and YopO specifically bind to Rac-1 and RhoA but not to Cdc42. *S. cerevisiae* EGY48 was cotransformed with pEG202 encoding full length YopO or YpkA and pJG4-5 encoding full length Rac-1, RhoA or Cdc42. Cotransformants were grown for 2 days at 27°C on leucine-deficient plates. A *S. cerevisiae* Leu^+ strain served as a positive, a *S. cerevisiae* Leu^- strain as a negative growth control.

yeast two-hybrid system. All four clones carried the complete Rac-1 gene. Since these inserts also encoded additional sequences besides the Rac-1 reading frame, full length Rac-1 was subcloned into pEG202 and tested again for interaction with YopO. Interaction could be confirmed. To further investigate whether the observed interaction is specific for Rac-1 or includes other members of the Rho family, we also tested interaction of YopO with RhoA as well as with Cdc42. The yeast two-hybrid system revealed an interaction of YopO with full length RhoA but not with full length Cdc42 (Fig. 1), although Cdc42 was found to be expressed in the yeast cells (data not shown). YpkA is the *Y. pseudotuberculosis* homolog of YopO (96% sequence identity). Therefore we supposed that it might also interact with Rac-1 and RhoA. In fact, the yeast two-hybrid system revealed an interaction between YpkA and Rac-1 or RhoA, respectively. As with YopO, binding of YpkA to Cdc42 could not be detected (Fig. 1).

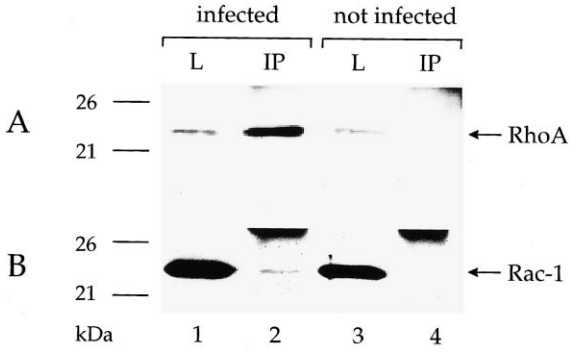

Fig. 2. YpkA interacts with RhoA and Rac-1 in vivo. COS cells were infected with WA-C (pTRANS, pCJYE138-*ypkA*) (lanes 1 and 2) or left uninfected (lanes 3 and 4) and lysed. The lysate was cleared by centrifugation and subjected to immunoprecipitation using anti-YpkA antibodies coupled to protein G-agarose. Coimmunoprecipitated proteins were subjected to SDS–PAGE followed by Western blot analysis using RhoA (A) or Rac-1 (B) antibodies. Eluates from the immunoprecipitation (IP) are shown in lanes 2 and 4. An aliquot of cleared lysate (L) was loaded in lanes 1 and 3 as a marker.

3.2. *YpkA interacts in vivo with Rac-1 and with RhoA but not with Cdc42*

To get further evidence for the physiological significance of the interactions observed in the yeast two-hybrid system, Rac-1, RhoA and Cdc42 were investigated for an interaction with YpkA in vivo. COS cells were infected with WA-C (pTRANS, pCJYE138-*ypkA*) (Table 1) and YpkA was immunoprecipitated using a polyclonal antibody. Western blotting revealed coimmunoprecipitation of YpkA with RhoA (Fig. 2A) and Rac-1 (Fig. 2B) but not with Cdc42 (data not shown). Although, under the experimental conditions used, more RhoA than Rac-1 was coimmunoprecipitated with YpkA, the coimmunoprecipitation qualitatively confirms the yeast two-hybrid results.

3.3. *Purified recombinant YpkA is not autophosphorylated*

YpkA was expressed recombinantly, purified and investigated for autophosphorylation activity. Therefore, recombinant YpkA was bound to antibodies, immobilized on protein G-agarose beads and subjected to an in vitro kinase reaction. As depicted in Fig. 3, recombinant YpkA was not autophosphorylated. However, addition of HeLa cell lysate to the assay resulted in significant phosphorylation of YpkA, indicating that a yet unidentified cytosolic host cell factor is required for phosphorylation of recombinant YpkA.

3.4. *YpkA directly interacts with Rac-1 and RhoA but not with Cdc42 in vitro*

The activity of small molecular weight GTPases of the Ras family depends on the type of guanosine nucleotide bound. When bound to GTP, they are active. Hydrolyzation of GTP to GDP inactivates their ability to bind effector molecules. A specificity for one of these two nucleotide forms could give an indication concerning the physiological function of the inter-

action between YpkA and RhoA or Rac-1. Thus, binding of YpkA to RhoA, Rac-1 and Cdc42 was investigated in different nucleotide loading states. The GTPases were always used in excess compared to YpkA to ensure abundant binding capacity of the GTPases. Since phosphorylation could have an impact on binding, recombinant YpkA was subjected to kinase reaction in the presence or absence of HeLa cell lysate. Recombinant YpkA was then added to GST-Rho, -Rac and -Cdc42 that were depleted of nucleotides or loaded with either GDP or 5′ guanylylimidodiphosphate, a non-hydrolyzable analog of GTP. As shown in Fig. 4A, nucleotide-depleted Rac-1 could no longer bind to YpkA whereas Rac-GDP and Rac-GTP did bind equally well. In contrast, RhoA bound to YpkA in the nucleotide-free form, in the GDP and in the GTP-bound state (Fig. 4B). These data further show that interaction of YpkA with either RhoA or Rac-1 is direct. Again, Cdc42 did not bind to YpkA in either form (Fig. 4C). Furthermore, the phosphorylation of YpkA achieved by the addition of HeLa cell lysate to the assay did not affect binding of YpkA to either RhoA or Rac-1, did not influence its binding preference for the GDP- or GTP-bound state of RhoA and Rac-1 and also could not support binding to Cdc42.

4. Discussion

In this study, we identified Rac-1 and RhoA as eukaryotic proteins interacting with YpkA of *Y. pseudotuberculosis* both in vitro and in vivo. The observed interaction of YpkA with Rac-1 and RhoA opens a wide array of possible cellular functions that could be influenced since Rac-1 and RhoA represent important regulators of cellular signaling [19–22].

Rho family GTPases represent molecular switches that cycle between a GTP-bound active and a GDP-bound inactive form. They are regulated by at least three groups of proteins: guanosine nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP; GDP dissociation inhibitors (GDIs) inhibit the dissociation of GDP from the GTPase, and GTPase activating proteins (GAPs) increase the hydrolysis activity of the GTPase [20,22].

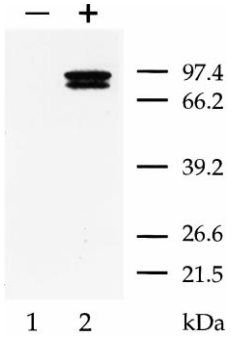

Fig. 3. Recombinant YpkA is not autophosphorylated in vitro but is phosphorylated after the addition of HeLa cell lysate. Recombinant YpkA was immunoprecipitated using a polyclonal YpkA antibody and immobilized on protein G-agarose beads. One sample was supplemented with HeLa cell lysate (lane 2) and immediately subjected to kinase reaction with 1 μCi [γ-^{32}P]ATP. The second sample (lane 1) was subjected to kinase reaction in the absence of HeLa cell lysate. Following SDS–PAGE, phosphorylation was visualized by autoradiography of the dried gel. (Phosphorylated as well as unphosphorylated recombinant YpkA always appeared as a doublet with the lower form likely representing a degradation product.)

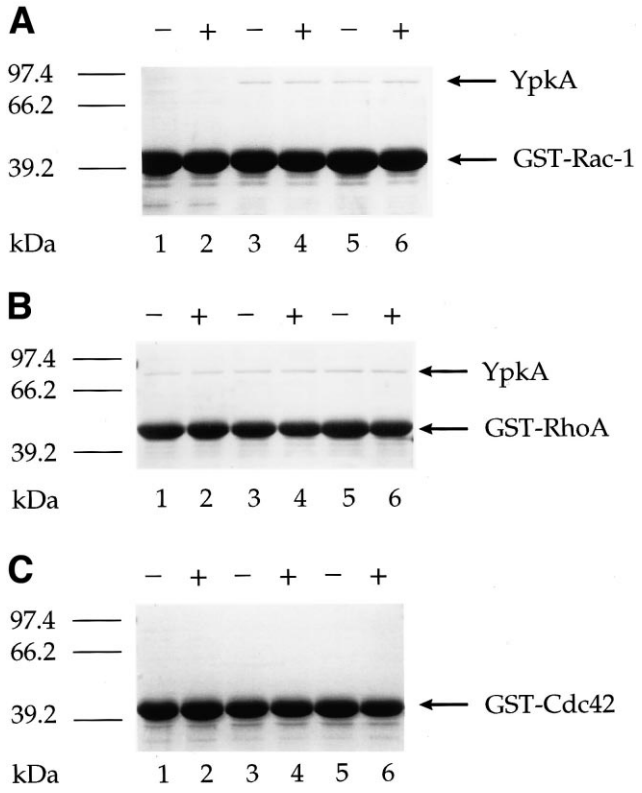

Fig. 4. Recombinant YpkA interacts with recombinant GST-RhoA and GST-Rac-1 independent of the GDP or GTP loading state. GST-Rac (A), GST-Rho (B) or GST-Cdc42 (C) were bound to glutathione-Sepharose beads, incubated in nucleotide depletion buffer for 20 min at room temperature, washed with GDP or GTP loading buffer and incubated for 25 min at room temperature. Recombinant YpkA which has been subjected to kinase reaction in the presence (+) or absence (−) of HeLa cell lysate was added to nucleotide-depleted GTPases (lanes 1 and 2), GDP-loaded GTPases (lanes 3 and 4) or GTP-loaded GTPases (lanes 5 and 6). Subsequently, samples were incubated, washed and separated by SDS–PAGE. Proteins are visualized by Coomassie stain.

Recently, it has been disclosed that the effector molecules SopE and SptP of *Salmonella typhimurium* interfere with the signaling pathways of Rac-1 and Cdc42 by acting as a GEF or GAP for these GTPases [23,24].

In vitro, we observed binding of YpkA to be equally tight to GST-Rac-GDP and GST-Rac-GTP whereas there was no binding to the nucleotide-depleted form of Rac. In contrast, YpkA bound equally well to the nucleotide-depleted, the GDP- and the GTP-bound form of RhoA. Since GEFs preferentially bind to the nucleotide-free form of GTPases and thereby stabilize this form [25], our data strongly suggest that YpkA does not function as a GEF on Rac or RhoA. GDIs and GAPs can bind to the GDP- and to the GTP-bound form of GTPases [26,27], which would be consistent with the binding characteristics observed for YpkA. However, the GDIs for Rho proteins so far described are exclusively localized in the cell cytosol whereas YpKA had been localized to the eukaryotic cell membrane [17]. Preliminary data further gave no evidence that YpkA has an inhibitory effect on Rho or Rac as would have been expected for a GAP (unpublished results).

Galyov et al. have demonstrated that secreted YpkA of *Y. pseudotuberculosis* exhibits autophosphorylating activity

[15] which might have an impact on its binding capabilities. In contrast, we did not detect any autophosphorylation of recombinant YpkA. This discrepancy might be explained by the fact that Galyov et al. investigated YpkA secreted by *Y. pseudotuberculosis* whereas we tested YpkA recombinantly expressed in *E. coli*.

The fact that recombinant, unphosphorylated YpkA binds to RhoA and Rac-1 in vitro clearly shows that phosphorylation is not crucial for binding. Interestingly, phosphorylation of recombinant YpkA can be achieved by the addition of HeLa cell lysate, suggesting the involvement of an additional eukaryotic factor. Very recently, it was shown by Juris et al. that actin is able to activate kinase activity of YpkA [28].

However, our data show that the phosphorylation of YpkA does not support or disturb binding of YpkA to RhoA and Rac-1, again excluding a crucial role of phosphorylation in influencing binding of these proteins. Furthermore, no indication was found that YpkA phosphorylates RhoA and Rac-1, even after activation with HeLa cell lysate (data not shown). However, it is conceivable that phosphorylation of YpkA might be important for downstream effects not identified in this study.

We conclude that the reported interaction of YpkA with Rac-1 and RhoA could provide an important molecular clue to explain the observed cytotoxic changes in HeLa cells [17] attributed to YpkA and to understand its essential role in mouse infection [15].

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