The Yersinia Virulence Factor YopM Forms a Novel Protein Complex with Two Cellular Kinases*

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Pathogenic Yersinia contain a virulence plasmid that encodes genes for intracellular effectors, which neutralize the host immune response. One effector, YopM, is necessary for Yersinia virulence, but its function in host cells is unknown. To identify potential cellular pathways affected by YopM, proteins that co-immunoprecipitate with YopM in mammalian cells were isolated and identified by mass spectrometry. Results demonstrate that two kinases, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1), interact directly with YopM. These two kinases associate only when YopM is present, and expression of YopM in cells stimulates the activity of both kinases. RSK1 is activated directly by interaction with YopM, and RSK1 kinase activity is required for YopM-stimulated PRK2 activity. YopM activation of RSK1 occurs independently of the actions of YopJ on the MAPK pathway. YopM is also required for Yersinia-induced changes in RSK1 mobility in infected macrophage cells. These results identify the first intracellular targets of YopM and suggest YopM acts to stimulate the activity of PRK2 and RSK1.

The bacterium Yersinia has three species that are pathogenic for humans. Yersinia pestis causes plague and is transmitted by fleas from rodent reservoirs to humans, frequently resulting in fatal infections. This bacterium is responsible for three plague pandemics and has been involved in two plague epidemics in western India as recently as 1944 (1). Two other species, Yersinia enterocolitica and Yersinia pseudotuberculosis, are also human pathogens, and infection with these species results in gastrointestinal disorders such as appendicitis, ileocolitis, and mesenteric adenitis. Contaminated food or water is the most common route of infection by these species, with swine serving as a major reservoir for these bacteria (2). Although the severity of the disease and the mode of transmission of pathogenic Yersinia species differs, they all target lymphoid tissues.

Yersinia spp. harbor a virulence plasmid of ~70 kb that encodes proteins termed Yops (for Yersinia outer proteins) that are either intracellular effectors or membrane proteins that create a delivery system for these effectors (3, 4). Six effector proteins from Yersinia (YopE, YopH, YopJ/P, YopM, YopT, and YpkA/YopO) are delivered into eukaryotic cells to inactivate the host immune response. YopE, YopH, YopT, and YpkA target the actin cytoskeleton through different mechanisms to prevent phagocytosis of the bacterium. YopJ inhibits the production of certain inflammatory cytokines by blocking the mitogen-activated protein kinase (MAPK) and NFκB signaling pathways. YopJ has also been implicated in apoptosis induction in macrophages through caspase-8 activation and inhibition of NFκB-induced transcription.

The effects of YopM on host response to Yersinia infection are largely unknown. However, YopM is required for full virulence of Y. pestis and Y. enterocolitica, as demonstrated in mouse infection models (5, 6). The YopM protein is an acidic, ~42-kDa protein composed almost entirely of leucine-rich repeat (LRR) motifs. The LRR motif is thought to be a protein-protein interaction motif and is present in multiple signaling molecules found both intracellularly and extracellularly (7). Initial studies suggested that YopM is secreted from the bacteria and interacts with thrombin to inhibit platelet aggregation (8). Subsequent studies demonstrated that YopM is translated into target cells via the type III secretion apparatus (9). Once inside the target cell, YopM moves from the cytoplasm to the nucleus via a vesicle-associated pathway (10, 11). A gene chip microarray analysis of Y. enterocolitica-infected macrophage cell lines suggests that YopM affects the expression of genes involved in cell cycle and cell growth (12). However, the mechanism by which YopM produces these effects on gene expression is unknown. This paper describes the identification of two intracellular targets of YopM, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney 293 cells were maintained in DMEM (Invitrogen) + 10% fetal bovine serum (FBS, Invitrogen) + penicillin/streptomycin (Invitrogen). Cells were transfected in 100-mm plates with 5 μg of DNA using FuGENE 6 (Roche Applied Science) at a ratio of 3:1 (FuGENE 6:DNA) according to the manufacturer’s instructions. Mouse macrophage J774A.1 cells were maintained in DMEM + 10% FBS (Hyclone) + 1 μM sodium pyruvate (Invitrogen).

Antibodies—The following antibodies were used in the experiments described: anti-FLAG M2 (Sigma), anti-PRK2 (Cell Signaling Technology) protein kinase C-like 2; RSK1, p90 ribosomal protein S6 kinase 1; FBS, fetal bovine serum; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PSD, post-source decay; LC, liquid chromatography; MS, mass spectrum; MBP, myelin basic protein; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; GST, glutathione S-transferase; CREB, cAMP-response element-binding protein.

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‡ The abbreviations used are: Yop, Yersinia outer protein; MAPK, mitogen-activated protein kinase; LRR, leucine-rich repeat; PRK2, protein kinase C-like 2; RSK1, p90 ribosomal protein S6 kinase 1; FBS, fetal bovine serum; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PSD, post-source decay; LC, liquid chromatography; MS, mass spectrum; MBP, myelin basic protein; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; GST, glutathione S-transferase; CREB, cAMP-response element-binding protein.
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In Vitro Pull-down Assay—The PRK2 and RSK1 kinases were in vitro transcribed and translated in the presence of [35S]methionine (PerkinElmer Life Sciences) using the TnT T7 Quick-coupled Transcription/Translation System (Promega) from linearized PRK2-V5 (Apel) or pcDNA3-HA-RSK1 (Neol) plasmid templates. Radiolabeled proteins were incubated with bacterially produced recombinant YopM-6xHis protein bound to nickel-agarose beads (Qiagen) in 50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40. Beads were subsequently washed with 50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 10 mM imidazole and run on SDS-PAGE. The gel was fixed in 40% MeOH, 10% glacial acetic acid, treated with Amplify (Amersham Biosciences), and dried onto Whatman paper. The gel was then exposed to film at ~80 °C with a screen.

Yersina Strains and Infection—The Y. pseudotuberculosis strains used in this study were derived from YP126 (serogroup O:3). YP22 has been described previously (22) and is deficient for the expression of YopE, YopH, and YopK proteins. The YP33 strain was derived from YP22 and contains a frameshift in yopM, disrupting the expression of the YopM protein. The YF35/yopM strain was constructed by introducing the IPTG-inducible YopM expression plasmid, p67N-YopM into YP33 through conjugation. Yersinia strains were grown in Luria broth at 26 °C, diluted 1:40 in Luria broth supplemented with 20 mM sodium oxalate and 20 mM MgCl2 (A600 ~0.1), and grown for 1 h at 26 °C. These cultures were then shifted to 37 °C and grown for an additional 2 h. For infections using YP35/yopM, YopM expression was induced with 0.1 mM IPTG for 2 h at 37 °C. Yersinia were washed in Hanks’ balanced salt solution (Invitrogen) and resuspended at A600 0.5 in Hanks’ balanced salt solution. J774A.1 cells were grown to 80–90% confluence in DMEM + 10% FBS + 1 mM pyruvate and infected for 2 h at 37 °C at a multiplicity of infection of 50.

RESULTS

YopM Associates with Two Protein Kinases in Mammalian Cells—The crystal structure of YopM has been solved and reveals that the YopM monomer has an amino-terminal a-helical hairpin followed by a curving repeat structure composed of a polyproline II helical conformation on the convex side. YopM is also shown to associate with two protein kinases in mammalian cells, YopM-6xHis and YopM-FLAG. YopM-6xHis was a gift of Don Huddler (University of Michigan). YopM-FLAG was a gift of Kim Orth (University of Chicago).

In vitro conditions were chosen to simulate the Yersinia virulence effector, a biochemical approach was used to identify proteins that interact with YopM in mammalian cells. In this approach, proteins that associate with YopM in transiently transfected cells were isolated by immunoprecipitation and identified by mass spectrometry. Lysates from 293 cells transfected with a FLAG-YopM expression vector or the empty FLAG vector were immunoprecipitated with anti-FLAG antibody, and the associated proteins were visualized by silver stain SDS-PAGE gels (Fig. 1A). Mass spectrometry

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The identity of the p80 and p78 proteins was determined by LC/MS/MS analysis (Fig. 1C). The MS/MS fragmentation spectra was used to search the NCBI non-redundant database for protein identification. Three MS/MS spectra from the p80 and p78 samples unambiguously identified these proteins as p90 ribosomal protein S6 kinase 1 (RSK1/p90 \( rsk \)/MAPKAP-K1), a member of the growth factor-regulated S6 serine/threonine kinase family (27, 28).

**PRK2, RSK1, and YopM Form a Novel Protein Complex in Cells**—The mass spectral identification of p120, p80, and p78 was confirmed by Western blot analysis of YopM-associated proteins (Fig. 2A). Lysates from 293 cells transfected with a FLAG-YopM plasmid or vector alone were immunoprecipitated with anti-FLAG antibody, and proteins were visualized by sequential Western blots using anti-FLAG, anti-PRK2, and anti-RSK1 antibodies. A single ~45-kDa protein corresponding to FLAG-YopM was detected in anti-FLAG Western blots. Anti-PRK2 Western blots reacted with a single band of 120 kDa co-immunoprecipitating with FLAG-YopM. Anti-RSK1 Western blots detected a YopM co-immunoprecipitating doublet of ~80 kDa. These results confirm the mass spectral identification of p120 as PRK2 and p80/p78 as RSK1.

The ability of YopM to interact with these cellular proteins was examined in *Yersinia*-infected cells to determine whether the interactions detected in transfected cells are also seen in the context of an infection. The macrophage cell line *J774A.1* was infected with *yopM* (YP22) or *yopM* (YP33) *Y. pseudotuberculosis* strains, immunoprecipitated with YopM polyclonal antisera, and the co-immunoprecipitation of RSK1 and PRK2 determined by Western blot. YopM co-immunoprecipi-
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The interaction of YopM with PRK2 and RSK1 was also assessed in vitro to determine whether these kinases bind to YopM directly. Recombinant YopM with a His6 tag was bound to nickel beads and incubated with radiolabeled in vitro transcribed/translated kinases (Fig. 2C). Proteins bound to the YopM beads were separated by SDS-PAGE, and the association of the kinases was visualized by fluorography. Both PRK2 and RSK1 bound to recombinant YopM in vitro. Deletion of the carboxyl terminus of these kinases abolished interaction with YopM in this assay (data not shown). These results, as well as results described in experiments to follow (Fig. 3), indicate that a region in the carboxyl-terminal half of these kinases is required for interaction with YopM. The in vitro binding data suggest a direct interaction between YopM and PRK2, as well as YopM and RSK1.

Further experiments were performed to determine whether YopM forms a single complex with these two kinases or two separate kinase complexes. Lysates from vector-transfected cells or cells transfected with FLAG-YopM were immunoprecipitated with anti-PRK2, anti-RSK1, or anti-FLAG antibodies, and proteins were visualized by Western blot (Fig. 2D). In vector-transfected cells, PRK2 and RSK1 do not co-immunoprecipitate, indicating that these two kinases normally do not associate in cells (lanes 2 and 3). However, in cells that express YopM, PRK2 co-immunoprecipitated both YopM and RSK1 (lane 4). RSK1 immunoprecipitated also showed interaction with both PRK2 and YopM (lane 5). These results demonstrate that YopM forms a single complex with PRK2 and RSK1 in transfected cells and that YopM induces the association of PRK2 and RSK1.

The subcellular localization of YopM is distinct from the other Yop effectors. YopM has been demonstrated to be injected into the cytoplasm of cells and to translocate to the nucleus via a vesicle-associated pathway (10, 11). PRK2 has been described as a cytoplasmic protein, whereas RSK1 has been demonstrated to shuttle between the cytoplasm and the nucleus of cells. To determine whether the subcellular localization of PRK2 and RSK1 is altered through association with YopM, the localization of the kinases was examined and compared with their localization in cells expressing YopM (Fig. 2E). Nuclear and cytoplasmic extracts were made from cells transfected with vector or FLAG-YopM, immunoprecipitated with anti-PRK2, anti-RSK1, or anti-FLAG antibodies, and visualized by Western blot. As a control for proper cell fractionation, the localization of a nuclear transcription factor, CREB, was also assessed in these extracts by Western blot. In vector-transfected as well as FLAG-YopM-transfected cells, PRK2 and RSK1 were localized primarily to the cytoplasm, with some nuclear localization as well. YopM was also detected in both cellular compartments,
with the majority of the YopM protein localized in the cytoplasm. These results demonstrate that PRK2, RSK1, and YopM localize to similar cellular locations, and association of the kinases with YopM does not redirect their localization.

Different Regions of PRK2 and RSK1 Are Required for YopM Association—In order to characterize further the protein interactions in the YopM complex, truncated kinase constructs were tested for their ability to co-immunoprecipitate with YopM (Fig. 3). The PRK2 domain structure contains three homologous repeat (HR1) regions in the amino terminus, followed by a central repeat region similar to the pseudosubstrate site of protein kinase C kinases (HR2), an SH3 domain-binding PXXP motif, and a carboxyl-terminal serine/threonine kinase domain (26). PRK2 deletion constructs were constructed in-frame with an amino-terminal GST tag and transfected into cells with FLAG-YopM. YopM complexes were immunoprecipitated with anti-FLAG antibody and Western-blotted with anti-GST antibody (Fig. 3A). YopM co-immunoprecipitated GST-PRK2 constructs containing the carboxyl-terminal amino acids 512–985 (Δ512N) and 648–985 (Δ648N). A weak interaction was also detected between YopM and GST-PRK2 amino acids 331–651 with long exposures. These results indicate that the major region of PRK2 required for interaction with YopM is between amino acids 648 and 985 which encompasses the kinase domain of PRK2.

The RSK1 domain structure consists of two distinct kinase domains separated by a linker region. Deletion constructs of RSK1 were constructed in-frame with an amino-terminal HA tag. The HA-RSK1 expression constructs were co-transfected into cells with FLAG-YopM, followed by anti-FLAG immunoprecipitation and Western blot analysis with anti-HA antibody (Fig. 3B). FLAG-YopM was able to co-immunoprecipitate full-length HA-RSK1 (FL) and the HA-RSK1 construct composed of amino acids 1–423 (Δ423C). Further truncation of HA-RSK1 abolished co-immunoprecipitation with YopM. These results suggest that interaction of RSK1 and YopM requires the presence of the linker region between the dual kinase domains of RSK1.

Association of PRK2 and RSK1 with YopM Enhances Their Kinase Activities—We have demonstrated that YopM forms a complex with two cellular kinases, PRK2 and RSK1. In vitro kinase assays were performed to determine the effect of complex formation on the kinase activity of PRK2 and RSK1 (Fig. 4). Endogenous kinases were immunoprecipitated from vector-transfected or cells transfected with FLAG-YopM and used in an in vitro kinase assay with [γ-32P]ATP and myelin basic protein (MBP) as substrate. As both RSK1 and PRK2 kinase activity is increased in cells grown in serum, transfected cells were serum-starved for 16 h prior to immunoprecipitation with rabbit IgG (c), anti-PRK2, anti-RSK1, or anti-Akt antibodies (Fig. 4A). The ability of both PRK2 and RSK1 to phosphorylate MBP was increased when YopM was expressed in cells. The kinase activity of Akt, a kinase not found in the YopM complex, was not affected by the expression of YopM. These results suggest that expression of YopM specifically activates PRK2 and RSK1.

The relative amounts of YopM expression required to activate PRK2 and RSK1 were investigated by in vitro kinase assays from cells transfected with increasing amounts of FLAG-YopM plasmid. Cells used for assessing PRK2 kinase activity were transfected with 0–5 μg of FLAG-YopM, whereas cells used in the RSK1 assays were transfected with 0–1 μg of FLAG-YopM, with the total amount of transfected plasmid maintained at 5 μg with the addition of vector plasmid. Cells were starved for 16 h and the endogenous PRK2 and RSK1 immunoprecipitated, and activity was assessed by in vitro ki-
YopM increased the kinase activity of RSK1 in vitro but did not significantly affect PRK2 kinase activity. The results suggest that YopM has a direct effect on RSK1 kinase activity and that the stimulation of PRK2 activity is due to an indirect mechanism.

The role of PRK2 and RSK1 kinase activities in the stimulation of the kinases by YopM was assessed using kinase-deficient mutants of PRK2 and RSK1 that contain mutations in critical lysines of their kinase domains that render them inactive (Fig. 5, B–D). In these experiments, cells were co-transfected with an EGFP-tagged YopM (EGFP-YopM), an HA-tagged RSK1 (HA-RSK, either wild-type or kinase-deficient), and a FLAG-tagged PRK2 (FLAG-PRK2, either wild-type or kinase-deficient). These cells were then serum-starved and components of the YopM complex immunoprecipitated, and kinase activity was analyzed by in vitro kinase assays.

The relative contribution of PRK2 and RSK1 kinase activity to the YopM complex was assessed by in vitro kinase assays with immunoprecipitated EGFP-YopM from transfected cells (Fig. 5B). In this assay, no kinase activity over control levels (lane 1) was seen in immunoprecipitates from cells transfected with both kinase-deficient PRK2 and RSK1 (lane 5), indicating that these are the only two kinases responsible for YopM complex phosphorylation of MBP. The kinase activity of the YopM complex was decreased by expression of kinase-deficient PRK2 (lane 3) and completely abolished by expression of kinase-deficient RSK1 (lane 4). These results demonstrate that both PRK2 and RSK1 kinase activity contribute to YopM-associated phosphorylation, and RSK1 activity is required for complex kinase activity.

The effect of RSK1 kinase activity on the activation of PRK2 by YopM was assessed through in vitro kinase assays of immunoprecipitated FLAG-PRK2 from the cells described above (Fig. 5C). As these assays were performed from serum-starved cells, PRK2 kinase activity is only significantly stimulated in cells expressing EGFP-YopM (lane 3). Immunoprecipitation of a kinase-deficient FLAG-PRK2 from cells expressing wild-type HA-RSK1 and EGFP-YopM still showed the ability (albeit a decreased ability when compared with wild-type) to phosphorylate MBP, probably due to co-immunoprecipitation of active RSK1 (lane 4). Immunoprecipitates of wild-type FLAG-PRK2 from cells expressing kinase-deficient HA-RSK1 and EGFP-YopM demonstrated weak kinase activity (lane 5), indicating that RSK1 activity affects the kinase activity of PRK2.

The activity of RSK1 was also assessed in cells expressing kinase-deficient FLAG-PRK2 and EGFP-YopM (Fig. 5D). Serum-starved transfected cells were immunoprecipitated with anti-HA antibody and immunoprecipitates used for in vitro kinase assays. The activity of HA-RSK1 from serum-starved cells was undetectable in this assay (lane 2), and expression of EGFP-YopM increased the kinase activity of HA-RSK1 (lane 3). HA-RSK1 activity was unaffected by expression of kinase-deficient FLAG-PRK2 (lane 4). Kinase reactions that included kinase-deficient HA-RSK1 demonstrated no significant kinase activity (lanes 5 and 6). These results indicate that FLAG-PRK2 kinase activity does not affect the EGFP-YopM stimulated activity of HA-RSK1. Taken together, these data support the model that YopM stimulates the kinase activity of RSK1, and the kinase activity of RSK1 stimulates the kinase activity of PRK2.

YopM Is Required for an Increase of RSK1 Phosphorylation during Yersinia Infection, Independent of the Effects of YopJ—In a *Yersinia* infection, multiple Yop effector proteins are injected into the cell cytosol. One of these effector proteins, YopJ, is a cysteine protease that has been shown to target the MAPK pathway by inactivating MAPK kinases (18) (Fig. 6A).
The Ras/MAPK pathway activates RSK1, through the phosphorylation of RSK1 by Erk1/2 (17). The Erk1/2 pathway is effectively blocked by the actions of YopJ, leading us to investigate whether YopM can activate RSK1 in cells that also express YopJ.

In vitro kinase assays were performed with endogenous RSK1 immunoprecipitated from serum-starved cells transfected with vector, FLAG-YopM, FLAG-YopJ, or both FLAG-YopM and FLAG-YopJ (Fig. 6B). The ability of RSK1 to phosphorylate MBP increased with the expression of FLAG-YopM (lane 3). This increased activity was not affected by co-expression of FLAG-YopJ (lane 5). These results demonstrate that YopM is able to activate RSK1 kinase activity independent of the effects of YopJ in cells.

We also examined the role of YopM in the activation of RSK1 in macrophage cells infected with yopM+ (YP22) or yopM− (YP33) Y. pseudotuberculosis strains (Fig. 6C). The macrophage cell line J774A.1 was left uninfected or infected with Y. pseudotuberculosis; whole cell lysates were made, and the mobility of the RSK1 protein was visualized by Western blot. In uninfected cells, RSK1 protein appears as multiple bands due to differences in migration of multiply phosphorylated forms of RSK1 (lane 1). Upon infection of the cells with YP22, RSK1 decreases in mobility, presumably due to an increase in phosphorylation of the kinase (lane 2). The apparent amount of RSK1 protein is less in lysates from YP22-infected cells due to unknown reasons but may be due to decreased reactivity of the antibody to the highly phosphorylated form of RSK1. Infection with YP33 results in the appearance of faster migrating (less phosphorylated) forms of RSK1 (lane 3).

To determine whether this change in mobility of RSK1 is due specifically to the actions of YopM, YopM expression was complemented in the YP33 strain by introduction of an IPTG-inducible YopM expression plasmid (YP33/yopM). The YP33/yopM strain produced a low level of YopM protein without
IPTG addition, and a significant amount of YopM protein was produced with IPTG stimulation (YP33/yopM/H11001) (Fig. 6C, lower panel). The molecular weight of Y. enterocolitica YopM produced by the expression plasmid (42 kDa) is different from YopM produced by Y. pseudotuberculosis YP22 (48 kDa). This heterogeneity of YopM protein size has been reported previously (29) with no known effect on YopM function. Expression of low levels of YopM modestly increased the amount of slower migrating forms of RSK1 (lane 4), and expression of high levels of YopM caused a significant decrease in RSK1 mobility, similar to infection with YP22 (compare lanes 2 and 5). These results indicate that infection of macrophage cells with Y. pseudotuberculosis causes a decrease in the mobility of RSK1 and that YopM protein expression is required for this change in RSK1 mobility.

**DISCUSSION**

In this study, we describe the identification of the first intracellular targets of the *Yersinia* virulence factor YopM. YopM is unique in comparison to the other Yop effectors, as it does not contain any obvious catalytic domains. The crystal structure of tetrameric YopM demonstrates a large surface for protein-protein interactions, suggesting a role as a protein scaffold (23). There are many examples of scaffolding proteins that regulate the activity of protein kinase pathways, including both the MAPK and protein kinase C pathways (30, 31). These scaffolding proteins are predicted to regulate not only the efficiency of kinase activation but also the specificity of substrates and the amplitude of the kinase cascade response (32). Our data suggest that non-catalytic YopM acts as a protein scaffold to recruit and activate proteins that have catalytic function, the serine/threonine kinases PRK2 and RSK1.

Functional studies of PRK2 suggest that it is a component of several signaling pathways involving the cytoskeleton, receptor tyrosine kinases, regulation of translation, and cell survival (Fig. 7). The kinase activity of PRK2 is stimulated by the binding of RhoA and has been demonstrated to alter the actin cytoskeleton (33, 34). Lipids have also been demonstrated to activate PRK2 and specifically negative phospholipids, such as cardiolipin (35). PRK2 is involved in signaling pathways activated by receptor tyrosine kinases through interaction with the adaptor proteins Nck and Grb4 (36, 37). PRK2 is also activated by interactions with MAPK kinase kinase 2, a kinase activated by the expression plasmid (~42 kDa) is different from YopM produced by Y. pseudotuberculosis YP22 (~48 kDa). This heterogeneity of YopM protein size has been reported previously (29) with no known effect on YopM function. Expression of low levels of YopM modestly increased the amount of slower migrating forms of RSK1 (lane 4), and expression of high levels of YopM caused a significant decrease in RSK1 mobility, similar to infection with YP22 (compare lanes 2 and 5). These results indicate that infection of macrophage cells with *Y. pseudotuberculosis* causes a decrease in the mobility of RSK1 and that YopM protein expression is required for this change in RSK1 mobility.

**Fig. 6.** YopM is required for an increase of RSK1 phosphorylation during *Yersinia* infection, independent of the effects of YopJ. A, schematic diagram of the Ras/MAPK pathway leading to the activation of RSK1. Shown are the kinases that YopJ inhibits and YopM activates. B, in vitro kinase assays were performed with rabbit IgG (c) or anti-RSK1 immunoprecipitates (IP) from serum-starved, transfected 293 cells with MBP included as a substrate. Amounts of RSK1 immunoprecipitated were detected by anti-RSK1 Western blot, and expression of FLAG-YopM and FLAG-YopJ was confirmed in anti-FLAG Western blot of cell lysates. Molecular mass markers are indicated on the left in kilodaltons. C, analysis of RSK1 migration from J774A.1 cells infected with *Y. pseudotuberculosis* strains by anti-RSK1 Western blot (upper panel). Expression of YopM was detected by anti-YopM Western blot of cell lysates (lower panel). *Yersinia* strains used were YP22 (yopM⁺), YP33 (yopM⁺), YP33/yopM (yopM⁻ + yopM plasmid), and YP33/yopM⁺ (yopM⁻ + yopM plasmid + IPTG).

**Fig. 7.** Conceptual model of YopM complex formation. In cells, RSK1 and PRK2 form separate signaling pathways with distinct activators and substrates. With the introduction of YopM into the cell, RSK1 and PRK2 are recruited into a new complex. Interaction of RSK1 with YopM activates its kinase activity, which in turn increases the kinase activity of PRK2. The activated YopM complex is now able to phosphorylate unidentified, possibly novel substrates.
in response to several stimuli, including growth factors and cross-linking of antigen receptors on T cells (15). In addition, PRK2 plays a role in initiating translation through phosphorylation of the mRNA cap binding factor, eIF4E (38). Finally, PRK2 affects cell survival pathways through regulation of Akt kinase activity (39, 40).

RSK1 is a member of the growth factor-regulated S6 serine/threonine kinase family (27, 28). These kinases have two non-identical kinase domains, and the amino-terminal kinase domain is thought to be responsible for phosphorylating substrates of RSK containing the consensus motif of (Arg/Lys)-X-Arg-X-(Ser/Thr) or Arg-X-(Ser/Thr) (where X is any amino acid). The Ras/MAPK pathway activates the RSK1 kinase through phosphorylation by Erk1/2 (Fig. 7). In addition, the carboxyl-terminal kinase domain of RSK1 and phosphoinositide-dependent protein kinase-1 (PDK1) also provide activating signals (28, 41). Upon activation, RSK1 has been demonstrated to phosphorylate substrates such as transcription factors (c-Fos, CREB, and SRF), transcriptional co-activators (CREB-binding protein), translational regulators (ribosomal S6 protein), cell cycle regulators (Myt1 and Na+/H+ exchanger isoform 1), apoptotic factors (BAD), and kinases (GSK3β and IKKα), suggesting roles for this kinase in cellular proliferation, translational regulation, cell survival, and glycolgen synthesis.

The kinases PRK2 and RSK1 have not been reported to interact under normal cellular conditions. Immunoprecipitation of PRK2 and RSK1 from transfected cells demonstrated that these kinases are recruited into a novel complex through interaction with YopM. The interaction of RSK1 and YopM was also seen in macrophage cells infected with Y. pseudotuberculosis. However, interaction of PRK2 with YopM was undetectable in infected cells, due to technical limitations of the antibodies used in these experiments and the level of PRK2 protein expression in these cells. The interaction of PRK2 and YopM from transfected cells is able to withstand multiple washes with buffers containing 1 M NaCl and 1% detergent, suggesting that it is a specific component of the YopM complex along with RSK1.

The formation of the YopM-kinase complex results in the activation of both RSK1 and PRK2. A model for activation of these kinases by YopM is diagramed in Fig. 7. In cells, RSK1 and PRK2 form separate signaling pathways with distinct activators and substrates. Cellular kinases RSK1 and PRK2 are recruited to YopM, and the direct interaction of RSK1 with YopM results in the activation of RSK1 kinase activity. The active RSK1 kinase then functions to stimulate the kinase activity of PRK2, resulting in an active YopM complex. PRK2 activation by YopM could be induced by phosphorylation of PKR2 by RSK1 or through phosphorylation-dependent changes of YopM. We have observed in transfected cells metabolically labeled with [32P]orthophosphate that YopM is phosphorylated (data not shown). Identification of the kinases responsible for this modification, sites of phosphorylation, and the effects of YopM phosphorylation will provide further insight into YopM function.

The cellular targets of PRK2 and RSK1 have been shown to be involved in several cellular functions, some of which overlap between the two kinases. Both of these kinases have been implicated in the regulation of proliferation, apoptosis, and translation (15, 27, 28, 38–40). We were unable to detect a change in cellular proliferation, translation, or the activity of some characterized targets of either PRK2 (Akt) or RSK1 (Bad, Jun, and CREB) in cells transfected with YopM (data not shown). We propose that the recruitment of PRK2 and RSK1 to YopM may alter their substrate specificity and/or recruit new substrates to these kinases, resulting in a novel signaling pathway (Fig. 7). This hypothesis will need to be tested by an analysis of the phospho-proteome of yopM− and yopM− Yersinia-infected cells. We anticipate that identification of the targets of the YopM complex described in this paper will provide further insight into the function of YopM.

In this study, we identify that YopM functions to stimulate endogenous RSK1 kinase activity. We observed increased activity of RSK1 in YopM-transfected cells by in vitro kinase assays and by a change in RSK1 mobility in Y. pseudotuberculosis-infected macrophage cells. Furthermore, the change of RSK1 mobility in infected cells was shown to be dependent on YopM, as complementation of a yopM− Yersinia strain with a YopM expression plasmid restored the slower mobility of RSK1. This apparent activation of RSK1 appears to be independent of upstream activators, as the strains used also translocate YopJ, a known inhibitor of the MAPK/Erk1 pathway (18, 22, 42). YopJ-independent activation of RSK1 was also demonstrated in vitro, as YopM is able to stimulate RSK1 kinase activity in cells expressing YopJ.

Activation of RSK1 by YopM is conserved function between species of Yersinia with heterogeneous YopM proteins. It was noted previously (29) that different strains of Y. pseudotuberculosis and Y. enterocolitica produce YopM proteins that vary from ~41 to ~55 kDa. The difference in size of these proteins is due to a triplication of a 60-amino acid region near the carboxyl terminus of YopM. No obvious effects on virulence have been noted between strains producing different sized YopM proteins; however, the effect these differences have on YopM function has been difficult to determine without a defined function for YopM. Infection with Y. pseudotuberculosis strains secreting an ~42-kDa Y. enterocolitica YopM protein or the endogenous ~48-kDa Y. pseudotuberculosis YopM protein both affected the mobility of RSK1 in macrophage cells. These data suggest a novel and conserved function for YopM, the activation of RSK1.

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