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To cite this version:

Martin Aepfelbacher, Claudia Trasak, Gottfried Wilharm, Agnès Wiedemann, Konrad Trulzsch, et al.. Characterization of YopT effects on Rho GTPases in Yersinia enterocolitica-infected cells.. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2003, 278 (35), pp.33217-33223. 10.1074/jbc.M303349200. hal-02679299

HAL Id: hal-02679299
https://hal.inrae.fr/hal-02679299
Submitted on 31 May 2020

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Characterization of YopT Effects on Rho GTPases in Yersinia enterocolitica-infected Cells*

Received for publication, April 1, 2003, and in revised form, May 9, 2003
Published, JBC Papers in Press, June 5, 2003, DOI 10.1074/jbc.M303349200

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Pathogenic yersiniae employ a type III secretion system for translocating up to six effector proteins (Yersinia outer proteins (Yops)) into eukaryotic target cells. YopT is a cysteine protease that was shown to remove the C-terminal isoprenoid group of RhoA, Rac, and CDC42Hs. Here we characterized the cell biological and biochemical activities of YopT in cells infected with pathogenic Yersinia enterocolitica. Bacterially injected YopT located to cell membranes from which it released RhoA but not Rac or CDC42Hs. In the infected cells RhoA was dissociated from guanine nucleotide dissociation inhibitor-1 (GDI-1) and accumulated as a monomeric protein in the cytosol, whereas Rac and CDC42Hs remained GDI-bound. Direct transfer of isoprenylated RhoA to YopT and RhoA modification could be reconstituted in vitro by guanosine 5′-3-O-(thio)triphosphate loading of a recombinant RhoA-GDI-1 complex. Finally, in macrophages infected with a Yersinia strain selectively translocating YopT podosomal adhesion structures required for chemotaxis as well as phagocytic cups mediating uptake of yersiniae were disrupted. These findings indicate that bacterially translocated YopT acts on membrane-bound and GDI-complexed RhoA but not Rac or CDC42, and this is sufficient for disruption of macrophage immune functions.

This work was funded by grants from the Deutsche Forschungsgemeinschaft (Ai 11 and projects B6 and B1 in SFB 413) to M. A. and J. H.. 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.

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§The abbreviations used are: TTSS, type III secretion system; Yops, Yersinia outer proteins; GDI, guanine nucleotide dissociation inhibitor; PIP2, phosphatidylinositol bisphosphate; ERMs, ezrin-radixin-moesin family; PBS, phosphate-buffered saline; GTP-\(\gamma\)S, guanosine 5′-3-O-(thio)triphosphate; GDP\(\gamma\)S, guanyl-5′-\(\gamma\)S triphosphate; HUVEC, human umbilical vein endothelial cells; GST, glutathione S-transferase.
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2 µg of YopT for 30 min at 37 °C. For GST pull-down RhoA-GDI complex (25 µg of protein/10 µl) was loaded or not with 200 µM GTP-γ-S and treated or not with 100 µM PIP2 as described above. Thirty µg of GST or 100 µg of GST-YopT were bound to 50 µl of glutathione-Sepharose beads in a buffer containing 20 mM Tris, pH 7.5, 1 mM EGTA, 3 mM MgCl2, and 1 mM dithiotreitol for 2 h at 4 °C and added to the samples of RhoA-GDI complex for 45 min at 37 °C. Beads were washed 5 times with PBS containing 0.1% Nonidet P-40 and boiled in Laemmli sample buffer, and proteins were run on polyacrylamide gels. GST pull down from cytosol was performed identically except that 1 mg of HeLa cell cytosol was used as a source for RhoA-GDI complex.

### RESULTS

**A Yersinia Strain That Translocates Selectively YopT**—We have previously shown that infection of cells with YopT-expressing but not with YopT-knockout Y. enterocolitica causes modification and membrane release of the GTP-binging protein RhoA (16). To investigate YopT in the absence of other Yops known to affect Rho GTPases, we constructed a strain named WAC(pYVO8) that translocates YopT as sole effector (Table I). To this end, Yersinia strain WAC that had been cured of the virulence plasmid pYVO8 was recombined with two plasmids, one containing genes encoding the complete type III secretion and translocation machinery (lcr region) and the other carrying the genes encoding YopT and its chaperone SycT of pYVO8. To test WAC(pYLCR), HUVEC were infected with this strain, stimulated with thrombin, and then analyzed for formation of actin stress fibers. As control, HUVEC were infected with strain WAC(pYLCR+T), which expresses full-length YopT fused to the N-terminal 138 amino acids of YopE in addition to wild type YopH, -O, -P, -E, -N (16). In contrast to strain WAC(pYLCR), whereas stress fiber formation was blocked efficiently in cells infected with WAC(pYLCR+T), overlay of actin and bacterial staining shows adherence of a similar number of bacteria.

![Image](http://www.jbc.org/)

**Fig. 1. Disruption of stress fibers in endothelial cells infected with a Yersinia strain translocating YopT.** HUVEC were infected for 2 h with WAC(pYLCR) or WAC(pYLCR+T) at 50–100 bacteria/cell and then stimulated with thrombin (1 unit/ml for 2 min). HUVEC were double-stained for actin and bacteria as described under “Experimental Procedures.” Actin stress fibers were readily formed in cells infected with WAC(pYLCR), whereas stress fiber formation was blocked efficiently in cells infected with WAC(pYLCR+T). Overlay of actin and bacterial staining shows adherence of a similar number of bacteria.

**Table I** Y. enterocolitica strains used in this study

| Strains and plasmids | Relevant genotype/phenotype | Reference No. |
|----------------------|-----------------------------|---------------|
| WAC(pYVO8)          | Serotype O8; clinical isolate WA-314 harbouring virulence plasmid pYVO8 | 20 |
| WAC(pYVO8+ΔT)       | Deficient in YopT production; insertional inactivation of YopT gene | 16 |
| WAC(pYVO8+ΔT+T)     | WAC(pYVO8+ΔT) complemented in runs with pACYC184 plasmid encoding YopT | 16 |
| WAC(pYLCR)          | Mutant strain containing the lcr region and yadA on vector pLAfRII; no effector Yop | 15 |
| WAC(pYLCR+T)        | WAC(pYLCR) complemented with plasmid pACYC184 encoding YopT and SycT; translocates functional YopT into cells | This study |

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**Yersinia YopT Effects on RhoA**

2 µg of YopT for 30 min at 37 °C. For GST pull-down RhoA-GDI complex (25 µg of protein/10 µl) was loaded or not with 200 µM GTP-γ-S and treated or not with 100 µM PIP2 as described above. Thirty µg of GST or 100 µg of GST-YopT were bound to 50 µl of glutathione-Sepharose beads in a buffer containing 20 mM Tris, pH 7.5, 1 mM EGTA, 3 mM MgCl2, and 1 mM dithiotreitol for 2 h at 4 °C and added to the samples of RhoA-GDI complex for 45 min at 37 °C. Beads were washed 5 times with PBS containing 0.1% Nonidet P-40 and boiled in Laemmli sample buffer, and proteins were run on polyacylamide gels. GST pull down from cytosol was performed identically except that 1 mg of HeLa cell cytosol was used as a source for RhoA-GDI complex.

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Peared in the cytosol of cells infected with Yersinia strains translo-
ating YopT, namely WAC(pYVO8), WAC(pYVO8△T+T), or
WAC(pYLCLR+T) (Fig. 3A). It can be seen from the relative propor-
tion between the lower and upper RhoA bands that WAC(pYLCLR+T) and WAC(pYVO8) induced a partial (about 70%)
RhoA modification, whereas WAC(pYVO8△T+T) modified all of
the RhoA. This phenomenon is likely due to the modest overpro-
duction of YopT by strain WAC(pYVO8△T+T) was loaded. B, HeLa
cells were not infected (Co) or infected with strain WAC(pYLCLR+T) for
2 h (Inf.), and cellular membranes were prepared as described under
"Experimental Procedures." Membranes were investigated by Western
blot (WB) using anti-RhoA, anti-CDC42Hs, or anti-Rac antibody. C,
suspensions of washed HeLa cell membranes were not treated (Control)
or treated with recombinant GST-YopTC139S or GST-YopT as de-
scribed under "Experimental Procedures." Membranes (Mem) and su-
pernatants (Sup) were separated by ultracentrifugation and tested for
the presence of the indicated proteins by Western blot.

and an upper band, which comigrated with the observed lower and
upper bands of GDI, respectively. The two Rac1 bands were main-
tained, and no additional Rac1 band appeared upon infection of
cells with WAC(pYLCLR+T) (Fig. 3B). Like RhoA, CDC42Hs was
detected in a lower band comigrating with GDI-1. No additional
CDC42Hs band appeared upon infection of cells with WAC(pYLCLR+T) (Fig. 3B). Together these results suggest that
YopT leads to specific disruption of the RhoA-GDI-1 association.

To test whether the YopT-modified RhoA is monomeric or bound
to another protein, cytosol of control or infected HeLa

Reconstitution of YopT-induced RhoA Modification Using
Purified RhoA-GDI Complex—As a first step to investigate
whether YopT can directly act on RhoA complexed to GDI, we
Yersinia YopT Effects on RhoA

RhoA in complex with GDI can be GDP- or GTP-bound. Whereas the RhoA-GDP-GDI complex is highly stable, loading of GTP into the complex was shown to promote translocation of RhoA to liposomes and cell membranes (25, 26). We therefore tested the effect of GTPγS or GDPγS loading on the ability of YopT to modify RhoA. The results in Fig. 5B clearly show that GTPγS loading into RhoA-GDI complex enabled YopT to modify RhoA, whereas GDPγS loading had no effect.

Last, we wanted to show direct transfer of RhoA from GDI to YopT. For this purpose we performed pull-down experiments using the “substrate trapping” GST-YopTC139S protein. The results depicted in Fig. 6 indicate that RhoA directly translocated from recombinant RhoA-GDI complex to YopT C139S in the presence but not in the absence of GTPγS. It was also interesting to note that upon PIP2 treatment, RhoA did not transfer to YopTC139S. Moreover, when the complex was treated with both PIP2 and GTPγS, only minor transfer of RhoA to YopTC139S occurred. Similar results were obtained when crude cytosol from HeLa cells was taken as a source of RhoA-GDI complex (Fig. 6). Unfortunately GDI-1 from the recombinant complex and GDI from cytosol shows unspecific binding to native glutathione beads and to GST-coated or GST fusion protein-coated beads irrespective of the washing procedure. However, because the amount of GDI associated with GST-YopTC139S-coated beads was not higher than that associated with GST-coated beads, one can conclude that YopT does not bind the RhoA-GDI-1 complex but to RhoA alone (data not shown).

**Fig. 5.** YopT modifies RhoA from cytosolic and recombinant RhoA-GDI complex. A, control cytosol and cytosol treated with GST-YopT or GST-YopTC139S was subjected to non-denaturing gel electrophoresis (NATIVE) and SDS/PAGE followed by anti-RhoA Western blot (WB). In the SDS-polyacrylamide gel YopT-modified RhoA shows a slightly higher mobility. B, recombinant RhoA-GDI complex was loaded or not with GTPγS or GDPγS as indicated. Thereafter, the complex was treated with YopT and PIP2 where indicated, and samples were analyzed by non-denaturing gel electrophoresis followed by anti-RhoA Western blot. Treatment of the RhoA-GDI complex with GTPγS or PIP2 enabled YopT to modify RhoA (lower arrow, unmodified RhoA; upper arrow, modified RhoA).
We conclude that GTP loading allows prenylated RhoA to transfer from GDI to YopT. In comparison, although PIP2 treatment allows YopT to modify RhoA in the GDI complex, high affinity binding of RhoA to YopTC139S does not seem to take place under these conditions.

Effects of YopT on Macrophage Immune FUNCTIONS—Among the most important target cells of enteropathogenic yersiniae in vivo are macrophages. The concerted action of the effector YopA is thought to block macrophage phagocytosis and chemotaxis as part of the immune-escape strategy of Yersinia (2). We therefore tested the effect of YopT on formation of cytoskeletal structures associated with phagocytosis and chemotaxis. It was shown that actin-rich phagocytic cups triggered by the surface adhesin invasin mediate phagocytosis of yersiniae (23). Using human macrophages we found that microinjection of GST-YopT or infection with WAC(pYLCR) infected human macrophages were infected with WAC(pYLCR) or WAC(pYLCR+T) for 2 h and then stained for actin using rhodamine phalloidin. Podosomes appearing as actin dots were disrupted by infection with WAC(pYLCR+T) but not with WAC(pYLCR). WAC(pYLCR+T) infected cells demonstrated pronounced ruffling (arrows).

RhoA is found in the plasma membrane, whereas the majority of Rac and CDC42 are on endosomal and Golgi membranes, respectively (28). In infected cells and upon the addition of recombinant YopT to cytosol, RhoA was released from GDI, but Rac and CDC42 remained GDI-bound. Considering that in our cell infection model the amount of translocated YopT is already at or above the values that could be obtained during human Yersinia infection, one may assume that YopT is specific for RhoA in vivo.

Using purified RhoA-GDI-1 complex we found that YopT could not cleave RhoA unless the complex was treated with PIP2 or GTPγS. In an earlier study PIP2 was shown to greatly increase the ability of C3-transferase of Clostridium botulinum to ADP-ribosylate RhoA in GDI complex (29). Another study showed that loading of GTPγS onto recombinant RhoGDI complex promoted translocation of RhoA to liposomes (25). However, because transfer of prenylated RhoA to YopT could only be detected after GTPγS loading but not after PIP2 treatment, these two mechanism of “loosening” the RhoA-GDI association seem to be different. Potentially PIP2 displaces the prenyl group of RhoA from its binding pocket in the GDI molecule, and YopT can then attack the partially opened RhoA-GDI complex. Yet the affinity of YopTC139S for this complex may be too low for pull down.

Combining our data and data from recently published work (17, 18), we propose the following scenario of YopT action. Upon infection of cells with yersiniae, the YopT protein is translocated to the plasma membrane, where it binds and cleaves RhoA, thereby releasing it from the membrane. Membranous YopT, in cooperation with the membrane lipid PIP2 and/or Rho guanine nucleotide exchange factors, can also attack the high affinity soluble RhoGDI complex in the cytosol. The cleaved

![Fig. 6. GTP loading allows RhoA to transfer from RhoA-GDI complex to YopT.](Image)

![Fig. 7. YopT disrupts phagocytic cups and podosomal adhesion structures in human macrophages.](Image)
Yersinia YopT Effects on RhoA

RhoA accumulates in the cytosol and is unable to associate with GDI or translocate to membranes. Moreover, after modification by YopT, the RhoA can no more associate with effector proteins (30). Thus, as a consequence of the multiple functional defects of YopT-modified RhoA, the latter most likely cannot fulfill signaling functions. However, we can already notice a RhoA negative phenotype, i.e. disruption of actin stress fibers, when just a part (30–50%) of RhoA is modified by YopT (16). Therefore, the YopT-modified RhoA may have dominant negative activity, as was proposed recently for a RhoA form in which the C-terminal polybasic region was cleaved off by calpain (31).

Recently the contribution of individual Yops to the antiphagocytic function of yersiniae was evaluated (32). It was found that bacteria lacking YopT were less resistant to phagocytosis. We show here that YopT can block the formation of actin-rich phagocytic cups induced by the Yersinia adhesion invasin. This YopT effect is rather slow, requiring at least 15–30 min of infection, but due the nature of the modification it is long-lasting. In comparison, YopE also disrupts invasin-triggered phagocytic cups, which starts immediately after bacteria cell contact but is rather transient. It seems likely, therefore, that YopE and YopT act synergistically in antiphagocytosis by inducing immediate and prolonged inhibition of Rho GTPases, respectively. Considering that YopT, YopE, and YopO act on overlapping sets of Rho GTPases, more examples of their cooperation on the molecular level can be expected in the future. Last, we can detect in cells a form of RhoA that has an identical activity, as was proposed recently for a RhoA form in which the C-terminal polybasic region was cleaved off by calpain (31).

Acknowledgments—We thank Norbert Zanker for expert technical assistance and Feng Shao and Jack Dixon for gifts of YopT expression constructs.

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*A. Wiedemann and M. Aepfelbacher, unpublished results.*
Characterization of YopT Effects on Rho GTPases in Yersinia enterocolitica-infected Cells

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J. Biol. Chem. 2003, 278:33217-33223.
doi: 10.1074/jbc.M303349200 originally published online June 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303349200

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