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Endoproteolytic Processing of RhoA by Rce1 Is Required for the Cleavage of RhoA by *Yersinia enterocolitica* Outer Protein T

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The bacterial toxin *Yersinia* outer protein T (YopT) is a cysteine protease that cleaves Rho GTPases immediately upstream of a carboxyl-terminal isoprenylcysteine. By clipping off the lipid anchor, YopT releases Rho GTPases from membranes, resulting in rounding up of mammalian cells in culture. The proteolytic activity of YopT depends on the isoprenylation of the cysteine within the carboxyl-terminal CaaX motif, a reaction carried out by geranylgeranyltransferase type I. The CaaX motif (where "a" indicates aliphatic amino acids) of Rho proteins undergoes two additional processing steps: endoproteolytic removal of the last three amino acids (i.e., –aaX) by Rce1 (Ras-converting enzyme 1) and methylation of the geranylgeranylcysteine by Icmt (isoprenylcysteine carboxyl methyltransferase). In in vitro experiments, RhoA retaining –aaX cannot be cleaved by YopT. Nothing is known, however, about the influence of Rce1-mediated removal of –aaX on the activity of YopT in living cells. We hypothesized that Rce1-deficient mouse fibroblasts, in which the geranylgeranylated Rho proteins are not endoproteolytically processed, would be resistant to YopT. Indeed, this was the case. Microinjection of recombinant YopT into Rce1-deficient fibroblasts had no impact on the subcellular localization of RhoA and no impact on cell morphology. To determine if carboxyl methylation is also required for YopT action, we microinjected YopT into *Icmt*-deficient fibroblasts. In contrast to the results with *Rce1*-deficient cells, YopT cleaved RhoA and caused rounding up of the *Icmt*-deficient cells. Our data demonstrate that *Rce1*-mediated removal of –aaX from isoprenylated Rho GTPases is required for the proteolytic activity of YopT in living cells, whereas carboxyl methylation by Icmt is not.

Rho proteins are key regulators of a wide variety of cellular functions, including regulation of actin structure, integrin signaling, and phospholipid signaling (12). Rho proteins are also important for the regulation of endocytosis, secretion, control of transcription, cell cycle progression, and cell transformation (for reviews see references 2, 8, 14, and 19). Like all members of the Ras superfamily of small GTPases, Rho GTPases cycle between the GDP-bound inactive and GTP-bound active forms. The exchange of GDP for GTP is catalyzed by guanosine nucleotide exchange factors. Inactivation of Rho results from hydrolysis of bound GTP, a process that is stimulated by GTPase-activating proteins (for a review see reference 13).

Rho proteins are posttranslationally modified by isoprenylation of the CaaX (where "a" indicates aliphatic amino acids) box cysteine at the carboxyl terminus, a reaction catalyzed by geranylgeranyltransferase type I. After geranylgeranylation, the terminal –aaX tripeptide is released by Ras-converting enzyme 1 (Rce1). Finally, the α-carboxylate anion of the newly exposed geranylgeranylcysteine is methylated by isoprenylcysteine carboxyl methyltransferase (Icmt) (20). It is widely accepted that isoprenylation is required for the proper activation and targeting of GTPases to membrane surfaces. Less is known, however, about the physiologic impact of the endoproteolysis and methylation steps. Recent studies, however, have shown that *Rce1* or *Icmt* knockouts in mice cause developmental lethal phenotypes (4, 6) and that the absence of these enzymes leads to the mislocalization of several small GTPases in embryonic fibroblasts (11).

Rho GTPases have attracted interest for studies on bacterial pathogenicity. A wide variety of pathogenic bacteria produce protein toxins that either activate or inactivate Rho GTPases. The *Yersinia enterocolitica* outer protein YopT cleaves the geranylgeranylated Rho GTPases RhoA, Rac, and Cdc42 directly upstream of the carboxyl-terminal geranylgeranylcysteine. In this way, the lipid anchor of the GTPases is cleaved off and the proteins are released from the membrane into the cytosol (16).

Recently, in vitro experiments revealed that isoprenylation of Rho GTPases is required for the recognition and cleavage of Rho proteins by YopT. Moreover, in vitro-prenylated RhoA that retained –aaX appeared to be resistant to cleavage by YopT (16). In view of the latter finding, we hypothesized that *Rce1*-deficient fibroblasts would be resistant to YopT-induced Rho inactivation and cell rounding. To test this hypothesis, we cultured mouse embryonic fibroblasts deficient in *Rce1* and studied YopT action after microinjection into living cells as well as by incubation of purified membranes with the toxin. We also analyzed the effect of injecting YopT into cells lacking Icmt. We provide evidence that Rce1 is required for the YopT-induced cleavage of RhoA and cell rounding, whereas carboxyl methylation by Icmt is not.

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MATERIALS AND METHODS

***Rce1*- and *Icmt*-deficient cell lines.** Spontaneously immortalized mouse embryonic fibroblasts lacking *Rce1* or *Icmt* were generated as described previously (5, 9). The genotype of the cells was confirmed by Southern blotting of genomic DNA.

Expression and purification of glutathione *S*-transferase (GST)-YopT. The YopT gene was amplified from the virulence plasmid pYV from *Y. enterocolitica* JB580v and cloned in frame into the expression vector pGEX2TGL (17). The glutathione fusion protein was expressed in *Escherichia coli* TG1 at an optical density of 0.8 at 29°C by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. After growing overnight, the cells were harvested and lysed by sonication in lysis buffer (20 mM Tris-HCl [pH 7.3], 10 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 5 mM dithiothreitol [DTT]) and purified by affinity chromatography with glutathione-Sepharose (Amersham Biosciences). The beads were washed five times with lysis buffer (without detergent and PMSF) and five times with wash buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl). GST-YopT was eluted from the beads with glutathione (10 mM in 50 mM Tris-HCl, pH 8.0) four times for 10 min and concentrated with a 30-kDa Centricon filter (Amicon).

Cell culture and microinjection. $Rce1^{+/+}$, $Rce1^{-/-}$, $Icmt^{+/+}$, and $Icmt^{-/-}$ cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin, and 1% streptomycin in humidified 5% CO_2 at 37°C. For microinjection, cells were seeded at a subconfluent density on glass coverslips (Cellocate; Eppendorf) and cultured overnight. GST-YopT (400 ng/μl in 50 mM Tris-HCl, pH 8.0, with or without Alexa-labeled rabbit immunoglobulin G [IgG] as indicated), buffer, or the catalytically inactive mutant GST-YopT C139A was microinjected into cells with an Eppendorf 5242 microinjector. Thirty minutes after injection, the cells were fixed in a formaldehyde solution (3.7% formaldehyde, 0.1% Tween 20 in phosphate-buffered saline [PBS]) and washed with PBS. Cells were then incubated with rhodamine-conjugated phalloidin (1 U/coverslip) for 1 h at room temperature and washed again with PBS. Actin fibers were visualized by fluorescence microscopy, and images were recorded (AxioCam HRm; Zeiss). Photographs of cells coinjected with a dye were taken 30 min after injection, once under fluorescence and once as phase contrast.

Virus production and transduction. The plasmid containing the human *RCE1* cDNA was a gift of Patrick Casey (Duke University Medical Center, Durham, N.C.). *RCE1* was cloned into the retroviral transfer vector pREX. The plasmids pMD-G and pMD-g/p were provided by R. Mulligan (Harvard Medical School, Boston, Mass.). The retroviral vector was produced by cotransfection of HEK-293T cells with pMD-G and pMD-g/p and the retroviral transfer vector using the calcium phosphate method. After 4 days, the supernatant fluid was collected and spun down to remove cellular debris. The virus-containing medium was filtered (0.22 μm) and then ultracentrifuged to concentrate the virus. $Rce1^{-/-}$ cells were infected with the virus in the presence of Polybrene (8 μg/ml). Transduction efficiency was monitored by fluorescence microscopy of the coexpressed green fluorescent protein.

Preparation of cell membranes. Subconfluent cells on 10-cm culture dishes were washed with ice-cold PBS and then scraped into 1 ml of mammalian lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF). Cells were disrupted by sonication (three times on ice), followed by centrifugation for 20 min at 1,000 × *g* to remove the nuclear fraction. After ultracentrifugation (60 min at 100,000 × *g*) of the supernatant fluid, the pellet containing the membrane fraction was resuspended in lysis buffer (without PMSF) and used in the membrane release assay.

Membrane release assay and Western blot analysis. Resuspended membrane fractions (100 μl) were incubated with 1 μM GST-YopT for 30 min at 37°C. After incubation, the sample was separated into membrane and soluble fractions by ultracentrifugation (60 min at 100,000 × *g*) and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% gel and transferred to a polyvinylidene difluoride membrane. After blocking with 5% nonfat milk for 1 h, the blots were incubated overnight with a polyclonal antibody against RhoA (119; Santa Cruz) and then for 1 h with a horseradish peroxidase-conjugated secondary antibody.

RESULTS

We and others have shown that the isoprenylation of Rho GTPases is required for substrate recognition by YopT (16, 18). Farnesylated as well as geranylgeranylated RhoA proteins are acceptable substrates, whereas V192Y RhoA, which cannot be isoprenylated, is not cleaved by YopT. Moreover, in vitro-prenylated RhoA retaining –aaX (terminating with –LVL and –LIL in human and mouse RhoA, respectively) was resistant to cleavage by YopT (16). To determine if Rce1-mediated cleavage of –aaX is required for substrate recognition in living cells, we microinjected recombinant YopT, buffer, or the catalytically inactive mutant YopT C139A as a negative control into $Rce1^{-/-}$ cells and assessed cell rounding. As expected, $Rce1^{+/+}$ cells rounded up within 15 min after microinjection of YopT and then detached from the dish. In contrast, the $Rce1^{-/-}$ cells showed no rounding after 15 min and retained normal morphology even 2 h after the injection (Fig. 1A and B). These data indicate that Rho GTPases that are isoprenylated but not further processed cannot be recognized and cleaved by YopT. To verify this result, we assessed whether YopT is capable of releasing RhoA from purified membranes of $Rce1^{+/+}$ or $Rce1^{-/-}$ cells. Thus, we prepared cell membranes, incubated them with YopT or buffer as a control, and separated soluble and membrane fractions by ultracentrifugation. We then analyzed the release of RhoA from the membranes, and the appearance of RhoA in the soluble fraction, by Western blotting. In line with the microinjection experiments, no RhoA was released by YopT from membranes of $Rce1^{-/-}$ cells, whereas efficient cleavage was observed in membranes from $Rce1^{+/+}$ cells (Fig. 1C). Further, we asked whether the YopT-catalyzed cleavage of RhoA could be restored by expressing human *RCE1* in the $Rce1^{-/-}$ cells with a retroviral system. As expected, the expression of human *RCE1* in the $Rce1^{-/-}$ cells resulted in the cleavage of RhoA by YopT (Fig. 1D). $Rce1^{-/-}$ cells expressing human *RCE1* also rounded up following microinjection of YopT (data not shown).

The loss of YopT action in *Rce1*-deficient cells could be due to –aaX sterically blocking the interaction of RhoA with YopT or, alternatively, the last step of the posttranslational modification, carboxyl methylation, being required for substrate recognition by YopT. To determine whether carboxyl methylation of RhoA is required for cleavage by YopT, we injected recombinant YopT or the catalytically inactive mutant YopT C139A into $Icmt^{-/-}$ cells and assessed cell rounding. When fully attached to the culture dish, $Icmt^{-/-}$ cells are flat and extremely stretched out (5), and the microinjection procedure itself leads to disruption of the cell membrane. We found, however, that it was possible to inject $Icmt^{-/-}$ cells shortly after seeding them (i.e., before they had become fully flattened out). Consequently, we injected the $Icmt^{-/-}$ and control $Icmt^{+/+}$ cells 2 h after seeding them onto the slides. To visualize the injected cells, an Alexa-labeled rabbit IgG was coinjected with YopT. Notably, $Icmt^{-/-}$ cells injected with the inactive mutant continued to flatten, whereas the YopT-injected cells rounded up and detached (Fig. 2A). These data indicate that the endoproteolytically processed but unmethylated Rho GTPases in $Icmt^{-/-}$ cells are cleaved by YopT. In line with these microinjection experiments, when purified membranes from $Icmt^{-/-}$ cells were incubated with YopT, RhoA was readily released into the soluble fraction (Fig. 2B). We conclude that carboxyl methylation of the geranylgeranylcysteine is not required for substrate recognition and cleavage by YopT.

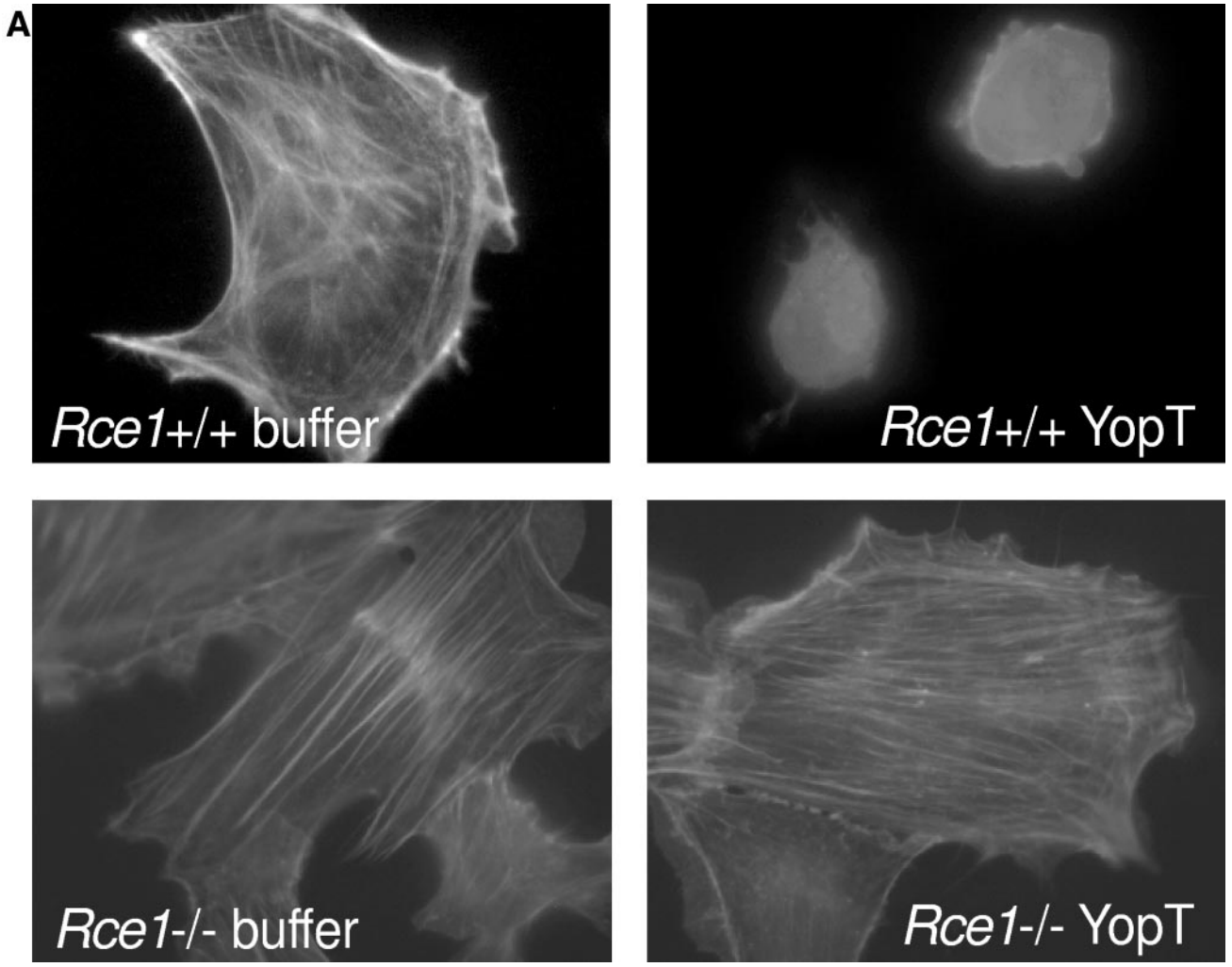
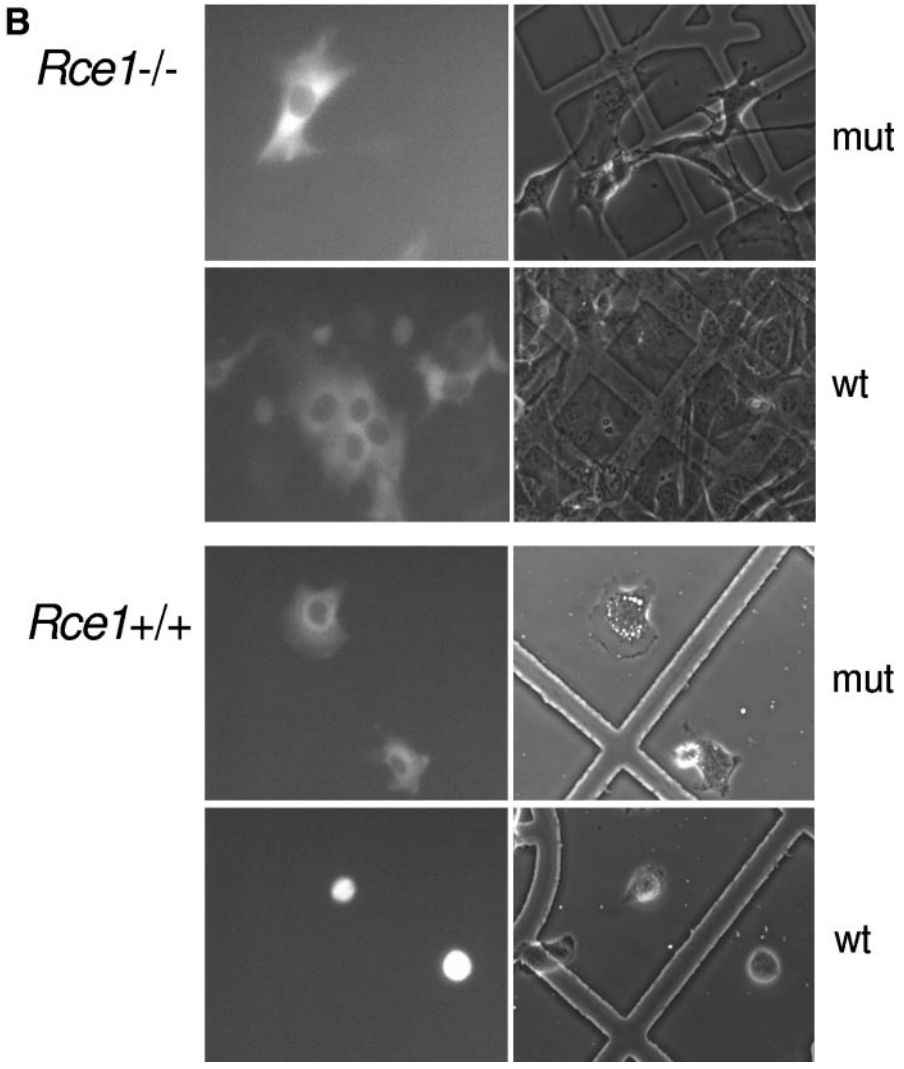


FIG. 1. *Rce1* deficiency prevents YopT-induced cell rounding and cleavage of RhoA. (A) $Rce1^{+/+}$ and $Rce1^{-/-}$ cells were injected with buffer or 400 ng/ml recombinant YopT as described in Materials and Methods. Thirty minutes later, the cells were stained with rhodamine-conjugated phalloidin and then viewed and photographed under a fluorescence microscope. (B) $Rce1^{+/+}$ and $Rce1^{-/-}$ cells were coinjected with an Alexa-labeled rabbit IgG (500 ng/ml) together with GST-YopT or the catalytically inactive mutant GST-YopTC139A (400 ng/ml, respectively) as described in Materials and Methods. Thirty minutes later, photographs of the cells were taken under fluorescence light and as phase contrast. (C) Membrane release assay demonstrating the appearance of YopT-cleaved RhoA in the soluble (S) fraction of $Rce1^{+/+}$ cells but not in the soluble fraction of $Rce1^{-/-}$ cells. Membranes of $Rce1^{+/+}$ and $Rce1^{-/-}$ cells were isolated and incubated with GST-YopT. After separation of membrane (P) and soluble (S) fractions by ultracentrifugation, the samples were analyzed for RhoA by Western blotting. Shown is a typical result of four independent experiments. As a consequence of the appearance of RhoA in the soluble fraction of $Rce1^{+/+}$ cells after incubation with YopT, there was a clear-cut reduction in the amount of RhoA in the membrane fraction. There was no reduction in the amount of RhoA in the membrane fraction of $Rce1^{-/-}$ cells after incubation with YopT. (D) Membrane release assay demonstrating the restoration of the YopT-catalyzed cleavage of RhoA in $Rce1^{-/-}$ cells by retroviral expression of human RCE1. Membranes of $Rce1^{+/+}$, $Rce1^{-/-}$, and "$Rce1^{-/-}$-*RCE1*" cells (i.e., $Rce1^{-/-}$ cells transfected with a human *RCE1* cDNA) were isolated and incubated with GST-YopT as described in Materials and Methods. After separation of membrane (P) and soluble (S) fractions by ultracentrifugation, the samples were analyzed for RhoA by Western blotting. We detected RhoA in the soluble fraction of $Rce1^{+/+}$ and $Rce1^{-/-}$-*RCE1* cells, whereas RhoA in $Rce1^{-/-}$ cells was exclusively found in the membrane fraction. The experiment was repeated three times with similar results. mut, mutant; wt, wild type; WB, Western blot.

DISCUSSION

This study demonstrates that Rce1-mediated release of the –aaX tripeptide from isoprenylated RhoA is required for substrate recognition by YopT, whereas carboxyl methylation by Icmt is not.

The *Yersinia* outer protein T (YopT) is a cysteine protease that cleaves Rho GTPases at their carboxyl terminus. It removes the geranylgeranyl lipid that anchors the GTPase to the plasma membrane. Recently, we and others showed that isoprenylation of the GTPase is absolutely required for cleavage by YopT (16, 18). It was further shown that the 13 carboxyl-terminal residues of RhoA are sufficient for cleavage by YopT. These data suggest that YopT recognizes both a sequence of basic amino acids and the isoprenylated cysteine. Moreover, it has been shown that YopT is targeted to the plasma membrane following its injection into cells by the bacteria. These data suggest that YopT is quite capable of reaching the substrate RhoA at the plasma membrane.



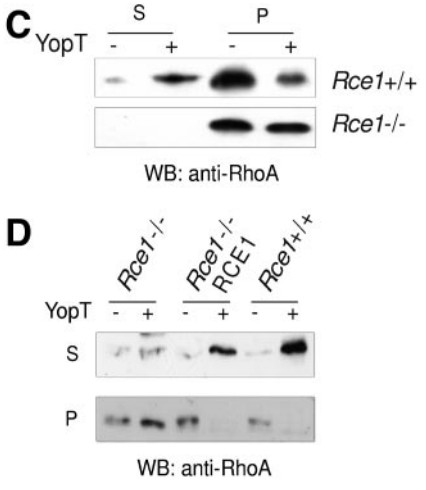


FIG. 1—*Continued.*

Using *Rce1*$^{-/-}$ fibroblasts, we showed that isoprenylated RhoA that has not undergone the Rce1-mediated removal of –aaX is not a substrate for YopT in living cells and, importantly, YopT-injected *Rce1*$^{-/-}$ cells do not round up and detach from the dish. We prepared membrane and cytosolic fractions from *Rce1*$^{-/-}$ cells and showed that RhoA containing –aaX is at least partly localized in the membrane fraction. (This result is entirely consistent with recent results from Michaelson et al. [11], which showed that Rce1-mediated processing affects the localization of farnesylated CaaX proteins but has little effect on the membrane targeting of geranylgeranylated CaaX proteins.) Even though RhoA was appropriately localized, the membrane-bound RhoA was not cleaved by YopT, indicating that it is not some mislocalization of the GTPase that blocks YopT action in these cells.

The loss of YopT action in *Rce1*-deficient cells could potentially be due to two factors: that –aaX sterically blocks the interaction with the toxin or that the last step of RhoA posttranslational modification, carboxyl methylation, is required for substrate recognition.

To determine if carboxyl methylation of RhoA is required for the recognition by YopT, we injected recombinant YopT into *Icmt*$^{-/-}$ cells and studied cell rounding. Using this assay, and by studying the membrane release of RhoA, we docu-

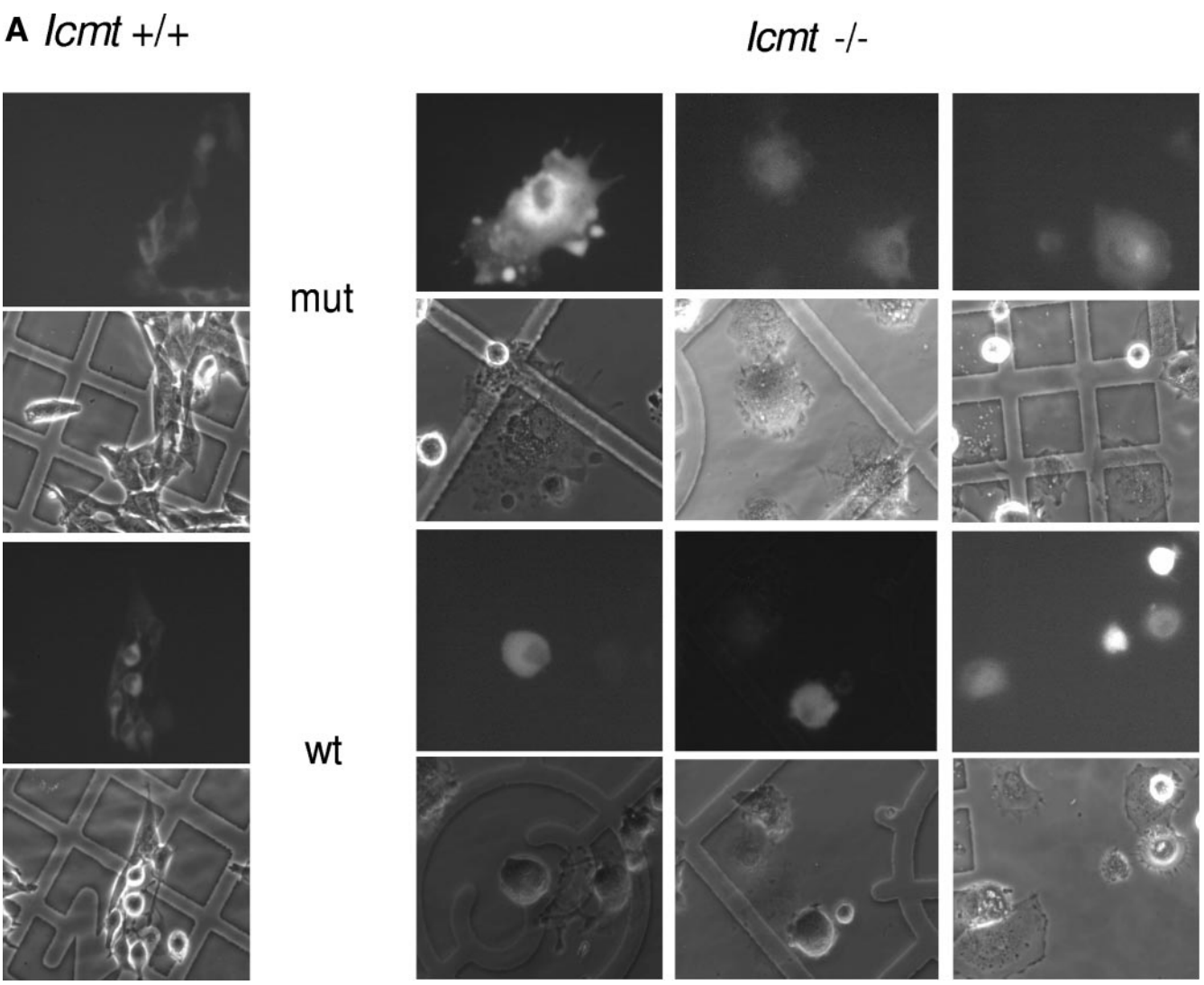


FIG. 2. Carboxyl methylation of RhoA is not required for cleavage by YopT. (A) The *Icmt*$^{-/-}$ cells are very flat and stretched out and are sensitive to the microinjection procedure when they are fully attached to the coverslip. To circumvent this issue, the *Icmt*$^{-/-}$ cells were injected before they were fully flattened out, 2 h after seeding *Icmt*$^{+/+}$, and *Icmt*$^{-/-}$ cells were coinjected with an Alexa-labeled rabbit IgG (500 ng/ml) together with GST-YopT or the catalytically inactive mutant GST-YopT C139A (400 ng/ml, respectively), as described in Materials and Methods. Thirty minutes later, photographs of the cells were taken once under fluorescence light and once as phase contrast. Note that the magnification of the objective used for *Icmt*$^{+/+}$ and *Icmt*$^{-/-}$ cells was 40× in all cases. Thirty minutes after injection of GST-YopT, the *Icmt*$^{+/+}$ cells have a rounded morphology. Injection of the catalytically inactive mutant GST-YopT C139A has no effect. YopT-injected *Icmt*$^{-/-}$ cells round up again after 30 min, whereas the control cells injected with the inactive mutant continue to attach and attain a flattened morphology. (B) Membrane release assay demonstrating YopT-cleaved RhoA in the soluble fraction (S) in both *Icmt*$^{+/+}$ cells (top panel) and *Icmt*$^{-/-}$ cells (lower panel). Membranes of *Icmt*$^{+/+}$ and *Icmt*$^{-/-}$ cells were isolated and incubated with GST-YopT as indicated. Following separation of membrane (P) and soluble (S) fractions by ultracentrifugation, samples were analyzed for RhoA by Western blotting. We detected RhoA from both *Icmt*$^{+/+}$ and *Icmt*$^{-/-}$ cells in the soluble fraction. The appearance of RhoA in the soluble fraction (S) was accompanied by a reduction in membrane-associated RhoA (P). All experiments were repeated five times with similar results. mut, mutant; wt, wild type; WB, Western blot.

mented that carboxyl methylation is not required for YopT recognition and cleavage of RhoA. Thus, it seems likely that the inability of YopT to cleave RhoA in *Rce1*$^{-/-}$ cells is due to the retention of the –aaX motif.

In view of the growing number of bacteria with antibiotic resistance, studying the exact action of bacterial toxins like YopT is potentially relevant to the treatment of bacterial infections. One of the pathogenic mechanisms in *Yersinia* infections is cleavage of Rho proteins by YopT (7). The influence of posttranslational modifications on other Rho-modifying toxins from *Yersinia* is unknown and should be studied. Thus, our studies with YopT provide a general example for future possibilities of directly counteracting the action of bacterial toxins in a patient with an acute bacterial infection. We propose that the toxicity of YopT with specific RCE1 inhibitor drugs (10, 15) might be quite feasible and without untoward side effects. Our previous experiments suggest that there is minimal toxicity when *Rce1* is inactivated. Previously, we generated a conditional allele for *Rce1* in mice and showed that *Cre*-mediated inactivation of *Rce1* had only barely perceptible effects on the growth of fibroblasts in culture (3). *Rce1* deficiency has no detectable effects on hematopoiesis (1), and *Cre*-mediated inactivation of *Rce1* has no detectable effect on the liver (8). Inactivation of *Rce1* adversely affected the heart, but that effect required months to be apparent (8). Thus, exemplified for YopT and an RCE1 inhibitor, future drugs might directly prevent the action of toxins by modifying substrate susceptibility in acute bacterial infections.

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