**Pseudomonas aeruginosa** ExoT Is a Rho GTPase-Activating Protein

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Received 25 April 2000/Returned for modification 20 June 2000/Accepted 31 July 2000

Transgenic intracellular expression of ExoT in CHO cells stimulated cell rounding and actin reorganization. Biochemical studies showed that ExoT was a GTPase-activating protein for RhoA, Rac1, and Cdc42. Together, these data show that ExoT interferes with Rho signal transduction pathways, which regulate actin organization, exocytosis, cell cycle progression, and phagocytosis.

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**Pseudomonas aeruginosa** is a gram-negative opportunistic pathogen affecting patients with cystic fibrosis, AIDS, severe burns, neutropenia, and ocular infections. Recent studies have shown that *P. aeruginosa* possesses a type-III system to deliver cytotoxins directly into eukaryotic cells (3). To date, four type-III cytotoxins have been identified: ExoS, ExoT, ExoY, and ExoU.

The enzymatic activity of exoenzyme S was purified from the culture supernatant of *P. aeruginosa* 388 as an aggregate composed of two proteins with apparent molecular masses of 49 kDa (ExoS) and 53 kDa (ExoT). Subsequent studies showed ExoS (8) and ExoT (14) were encoded by separate genes and had 76% primary amino acid homology. ExoS is a bifunctional cytotoxin that encodes two independent catalytic activities. The carboxyl terminus encodes a cytoxic FAS (factor activating exoenzyme S)-dependent ADP-ribose transferase activity (6) while the amino terminus stimulates the intrinsic GTPase activity of Rho GTases (4). ExoT also catalyzes a FAS-dependent ADP-ribosylation of eukaryotic proteins, but at a velocity that is only 0.2% of that of ExoS (9). Recently, Vallis et al. showed that delivery of ExoT by *P. aeruginosa* stimulates CHO cells to round (13).

ExoT-encoding plasmid (pEGFP-N1) and the indicator plasmid (pEGFP-N1) using 1 μg of actin) 

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engineered, subcloned into pET15b (pExoT78-237), and sequenced to confirm the absence of secondary mutations. ExoT (78-237) was expressed in E. coli BL21(DE3) as a His(6) fusion protein and purified by Ni-affinity chromatography as previously described (6). Purified ExoT(78-237) was dialyzed in 25 mM Tris, pH 7.6, plus 40% glycerol and stored at −20°C. ExoT(78-237) was determined to have an apparent molecular mass of 22 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and purity of about 85%. ExoT(78-237) possessed limited immunoreactivity to a polyclonal antibody that was prepared against ExoS (7).

The GAP activity of ExoT(78-237) was determined with a filter-binding assay (4). Rho GTPases were loaded with 10 μM [γ-32P]GTP. Intrinsic GTPase activity was initiated with the addition 3 mM MgCl2 and 1 mM GTP. After 5 min, the reaction mixtures were spotted onto a nitrocellulose filters, the filters were washed, and the remaining radioactivity was measured by scintillation counting. Stimulation of GTP hydrolysis by ExoT(78-237) or ExoS(78-234) was measured, using a concentration of ExoT or ExoS that stimulated the hydrolysis of less than 25% of the available Rho-GTP. Under these conditions the rate of stimulation of GTP hydrolysis by ExoS was linear for 5 min (4). The intrinsic rates of GTPase activities (in picomoles of GTP per minute per microgram of protein) were greatest for recombinant glutathione S-transferase–RhoA, followed by Rac1 and Cdc42 (data not shown). ExoT(78-237) stimulated the intrinsic GTPase activity of RhoA in a dose-dependent manner and at a rate similar to that stimulated by ExoS(78-234) (Fig. 2). Figure 2 also shows that ExoT(78-237) stimulated the GTPase activity of Rac1 and Cdc42.

Rho GTPases regulate numerous eukaryotic cellular functions, including actin cytoskeleton reorganization (5), exocytosis (1), transcription (12), and phagocytosis (2). Like other members of the family of Ras monomeric G proteins, Rho GTPase modulate cell physiology through their ability to cycle between an inactive GDP bound form and an active GTP bound form that is able to interact with downstream effectors. The Rho GTPases possess low intrinsic capacity to perform guanine nucleotide exchange or to catalyze the hydrolysis of GTP to GDP. These two activities are modulated by two types of eukaryotic protein, guanine exchange factors and GAPs. The ability of ExoT to express Rho GAP activity identifies it as a biochemical activity, which may contribute to the pathogenic potential of P. aeruginosa.
J.T.B. was supported by AI30162 from the NIH-NIAID. K.A. was supported by the DFG.

We thank Kristin J. Pederson for technical assistance during the study.

**ADDENDUM IN PROOF**

We acknowledge that Joanne Engel and coworkers have also observed that ExoT is a RhoGAP (B. I. Kazmierczak, T. S. Jou, K. Mostov, and J. N. Engel, Cell. Microbiol., in press).

**REFERENCES**


**FIG. 2.** ExoS and ExoT modulation of intrinsic activity of Rho GTPases. The intrinsic GAP activities of RhoA, Rac1, and Cdc42 were determined by filter-binding assay as described in the text. Nucleotide-free Rho GTPases were loaded with [γ-32P]GTP for 5 min at 37°C. The amount of GTP bound initially (t = 0) was determined, and intrinsic GTPase activity was initiated with the addition of MgCl2. After 5 min, the amount of GTP remaining bound to the Rho GTPase was determined, and the results are presented here as percentages of the amount of GTP bound initially (100%). The amount of GTP bound in the presence of the indicated amount of ExoS(78-234) or ExoT(78-237) was also determined after the addition of MgCl2. After 5 min, the amount of GTP remaining bound to the Rho GTPase was determined, and the results are presented here as percentages of the amount of GTP bound initially (100%). Typical GTP loading of RhoA was 0.6 to 1.0 mol of GTP bound per mole of RhoA. The data are means + standard deviations (error bars).