The N-terminal Domain of Pseudomonas aeruginosa Exoenzyme S Is a GTPase-activating Protein for Rho GTPases

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Pseudomonas aeruginosa exoenzyme S (ExoS) is a bifunctional cytotoxin. The ADP-ribosyltransferase domain is located within the C terminus part of ExoS. Recent studies showed that the N terminus part of ExoS (amino acid residues 1–234, ExoS(1–234)), which does not possess ADP-ribosyltransferase activity, stimulates cell rounding when transfected or microinjected into eukaryotic cells. Here we studied the effects of ExoS(1–234) on nucleotide binding and hydrolysis by Rho GTPases. ExoS(1–234) (100–500 nM) did not influence nucleotide exchange of Rho, Rac, and Cdc42 but increased GTP hydrolysis. A similar increase in GTPase activity was stimulated by full-length ExoS. Half-maximal stimulation of GTP hydrolysis by Rho, Rac, and Cdc42 was observed at 10–11 nM ExoS(1–234), respectively. We identified arginine 146 of ExoS to be essential for the stimulation of GTPase activity of Rho proteins. These data identify ExoS as a GTPase-activating protein for Rho GTPases.

Rho GTPases, Rho, Rac, and Cdc42 are involved in the regulation of the actin cytoskeleton by cell membrane-bound receptors and act as molecular switches in a large array of signaling processes (1, 2). Recent studies indicate that the GTPases are the preferred eukaryotic substrates of various bacterial protein toxins and exoenzymes (3, 4). C3-like exoenzymes (e.g. Clostridium botulinum exoenzyme C3) ADP-ribosylate RhoA, B, and C at asparagine 41, inhibiting the biological functions of the GTPases (5–7). Large clostridial cytotoxins (e.g. Clostridium difficile toxins A and B) monoglycosylate Rho threonine 37 and Rac and Cdc42 at threonine 35 (8, 9) and induce rounding up of cells and redistribution of the actin cytoskeleton. Escherichia coli cytotoxic necrotizing factors (CNF 1 and 2) and dermonecrotic toxin DNT of Bordetella species (10, 11, 18) activate Rho family GTPases by increasing the lifetime of the active GTP-bound form of the Rho protein. CNF and DNT deamidate and/or transglutaminate glutamine 63 of Rho (glutamine 61 of Rac and Cdc42), which inhibits the intrinsic and GTPase-activating protein (GAP)-stimulated GTP hydrolysis, resulting in a constitutively activated form of the GTPases.

Pseudomonas aeruginosa produces two ADP-ribosyltransferases: exotoxin A, which ADP-ribosylates elongation factor 2, or exoenzyme S (ExoS), which ADP-ribosylates Ras (13). ExoS (453 amino acids) is secreted and translocated into eukaryotic target cells by a type III secretion mechanism, which requires bacterial to eukaryotic cell contact. The ADP-ribosyltransferase activity by ExoS is dependent upon a eukaryotic cofactor (FAS, factor activating exoenzyme S), which is a member of the 14-3-3 protein family (14). Ras and several other proteins are preferred eukaryotic substrates of ExoS (15). ExoS ADP-ribosylates Ras at arginine 41 and arginine 128. ADP-ribosylation at arginine 41 blocks the activation of Ras by its guanine nucleotide exchange factor, thereby interfering with Ras-mediated signal transduction (16). The ADP-ribosyltransferase domain is located within the C-terminal domain (residues 234–453) of ExoS. Recently, transfection or microinjection of the N-terminal domain (residues 1–234) of ExoS elicited the reorganization of actin stress fibers, which resulted in destruction of the actin cytoskeleton and a rounded cellular phenotype (17).

The observation that CNF1, which activates Rho GTPases (11, 18, 19), reversed the disruption of actin stress fibers elicited by ExoS(1–234) (17), suggested that ExoS disrupted signal elements upstream of Rho GTPases or acted by the direct disruption of Rho GTPase function. Here, ExoS is shown to stimulate GTP hydrolysis by Rho, Rac, and Cdc42, indicating that ExoS acts as a GAP protein for Rho GTPases.

EXPERIMENTAL PROCEDURES

Purification of Full-length ExoS—Recombinant ExoS was cloned into pUCP and expressed in P. aeruginosa PA103. Recombinant ExoS was purified by gel filtration followed by ion exchange chromatography as described previously (20).

Cloning and Purification ExoS(1–234)—Construction of ExoS(1–234), consisting of amino acids 1 through 234 of ExoS, was performed by amplification of the vector ExoS234 (17) to introduce BamHI and EcoRI sites with the following primers.

ExoS(1–234) sense, GGATCCATGCATATTCAATCGCTTCAGCAG

ExoS(1–234) antisense, GAATTCGTCGGCCGATACTCTGCTGAC.

The polymerase chain reaction product was purified from an agarose gel and ligated into the pCR®Blunt II Vector. The proper construct was confirmed by DNA sequencing. The vector was transformed into E. coli BL21 (DE3) cells by heat shock at 42 °C.

GST fusion proteins were expressed in E. coli BL21 (DE3). Cells were cultured at 37 °C, and 0.2 ml isopropyl-β-D-thiogalactopyranoside (fi-
ExoS Is a RhoGAP

The various Rho-GAP proteins identified so far are known to stimulate the GTPase activity of RhoA about 20-fold. Whereas the half-time for the intrinsic rate of GTP hydrolysis by RhoA was about 5 min, in the presence of ExoS(1–234) about 80–90% of the GTP was hydrolyzed after 30 s. p50RhoGAP was also observed to stimulate GTP hydrolysis by RhoA at a similar rate (data not shown). This suggested that ExoS stimulated GTP hydrolysis by RhoA and acted like a GAP protein.

Substrate Specificity for the GAP Activity of ExoS—The specificity of ExoS to act as a GAP protein was tested in the presence of various small GTPases, including RhoA, Rac1, Cdc42, Ras, and Rap. Whereas ExoS(1–234) stimulated the GTPase activity of Rho, Rac, and Cdc42, the rate of GTP hydrolysis by Ras and Rap was not stimulated by ExoS(1–234) (Fig. 2). These data agreed with the earlier observation that the rounding phenotype elicited by ExoS(1–234) could be reversed by CNF1, which activates members of the Rho family of GTPases but not members of the Ras family of GTPases. The observation that the GTPase activity of all three members of the Rho family of GTPases (Rho, Rac, and Cdc42) were stimulated by ExoS was also consistent with our earlier observation that transfection with a dominant active form of Rho did not reverse the rounding phenotype elicited by ExoS (17). Since CNF1 blocked the effects of ExoS, the ability of ExoS(1–234) to stimulate the GTPase activity of recombinant dominant active RhoA(Q63E) was also tested. Consistent with the earlier reported reversal of ExoS(1–234)-induced cell rounding by CNF1, the GTPase activity of RhoA(Q63E) was not stimulated by the addition of 100 nM ExoS(1–234) (Fig. 2 and data not shown). CNF1 deamidates Gln-63 of RhoA, leading to the formation of Rho(Q63E). This constitutively activated form of Rho has been shown to be insensitive to p50RhoGAP-stimulated GTP hydrolysis. These data explain how the intoxication of cells with CNF1 reversed the rounding phenotype elicited by ExoS(1–234) (17).

The various Rho-GAP proteins identified so far are known to differ in their specificity toward Rho family GTPases. For example, whereas p50RhoGAP catalyzes the GTPase activity of...
Rho, Rac, and Cdc42 (22, 23), N-chimerin is specific for Rac, and Myr5 is specific for Rho and Cdc42 (24, 25). To extend the substrate preference for RhoA, Rac1, and Cdc42, the stimulation of GTPase hydrolysis by ExoS(1–234) was analyzed in a dose-dependent assay. To reduce the intrinsic rate of GTP hydrolysis and increase the resolution of the analysis, the assay was performed at 16 °C rather than 37 °C. As shown in Fig. 3, similar concentrations of ExoS(1–234) stimulated the GTPase activity for each GTPase, with a slight preference for Rac. This indicated that ExoS increased the GTP-hydrolyzing activity of all three Rho proteins with similar velocity.

Full-length Recombinant ExoS Stimulates the GTPase Activity of RhoA—To determine the GAP activity of a physiological form of ExoS, the ability of full-length ExoS to stimulate GTPase activity of RhoA was measured. Fig. 4 shows that full-length ExoS stimulated the GTPase activity of RhoA in a dose-dependent manner. Boiling ExoS for 10 min blocked the activity of the enzyme, consistent with this process being ExoS-mediated and not buffer-driven. Similar concentrations of full-length ExoS and of the fragment ExoS(1–234) were needed for the stimulation of the GTPase activity, which was consistent with the entire GAP domain residing within the N-terminal domain of ExoS.

Sequence Alignment with Known Rho GAPs—Due to its activity as a RhoGAP, the amino acid sequence of ExoS(1–234) and other known GTPase-activating proteins specific for Rho proteins were compared. Interestingly, little sequence similarity was observed among these enzymes. However, limited sequence similarity was observed between various Ras GAPs and ExoS (Fig. 5). Recent crystal structure analysis of Rho and Ras GAPs showed that these GTPase-activating proteins act by introducing an arginine finger into the GTPase, which is involved in stabilizing the intermediate state of GTP hydrolysis (26, 27). This arginine residue (arginine 146) is included within the region of amino acid similarity between the Ras GAPs and ExoS.

Arginine 146 of ExoS Is Required for the Stimulation of GAP by Rho—Following the sequence alignment shown in Fig. 5, arginine 146 was changed to lysine to determine if this amino acid is required for the expression of GAP activity by ExoS. ExoS(1–234)-R146K was expressed as a GST fusion protein, affinity-purified, and analyzed for the ability to activate the GTPase activity of RhoA in a filter binding assay. Consistent with this arginine contributing to the capacity of ExoS to stimulate GAP activity, 100 nM ExoS(1–234)-R146K did not accelerate the intrinsic GTPase activity of RhoA (Fig. 6). In contrast, a second mutated protein, ExoS(1–234)-R137K retained the ability to stimulate the GAP activity of RhoA.

The ability of proteins that act as GAPs is not unique to monomeric GTPases. The GTPase activity of the heterotrimeric G proteins is stimulated about 100-fold by a class of proteins termed the regulators of G protein signaling (27). G protein-signaling proteins and the GAP proteins share little primary amino acid homology and differ with respect to the presence of
an active-site arginine within the arginine finger motif. Although GAPs supply this arginine to stimulate GTP hydrolysis stimulated by monomeric GTPases, the active-site arginine is located within the heterotrimeric G protein, which accounts for the higher intrinsic GTPase activity of heterotrimeric G proteins relative to monomeric GTPases. Since the maximal GTPase activities of heterotrimeric G proteins and monomeric GTPases when stimulated by G protein-signaling proteins and GAP proteins, respectively, are similar, G protein-signaling proteins and GAPs must also stimulate GTPase activity through secondary interactions that are independent of the arginine finger motif. The observation that ExoS stimulated Rho GTPase activity to similar levels as observed with RhoGAP suggested that ExoS also interacted with Rho through the arginine finger motif and a secondary interaction that is independent of the arginine finger motif (27).

ExoS possesses two distinct domains, which interfere with eukaryotic signal transductions. The C terminus of ExoS (residues 234–453) comprises the ADP-ribosyltransferase domain, which ADP-ribosylates small molecular weight GTPases (28). Recent studies have shown that ExoS ADP-ribosylates Ras at arginine 41 and arginine 128 (13) and that the ADP-ribosylation at arginine 41 inhibits the interaction with its guanine nucleotide exchange factor (16). This inhibits Ras-mediated signal transduction within the cell. The N terminus of ExoS (residues 1–234) comprises the GAP domain, with arginine 146 participating in catalysis. Substrate analysis showed that the GAP activity of ExoS was comparable for the three subtypes of Rho GTPases, Rho, Rac, and Cdc42. This may relate to the role of the GAP activity of ExoS in microbial pathogenesis, since there appear to be two distinct mechanisms of macrophage-mediated phagocytosis that are controlled by the Rho GTPases (12, 29). Type 1 phagocytosis utilizes the immunoglobulin receptor and is mediated by Cdc42 and Rac, whereas type II phagocytosis utilizes the complement receptor and is mediated by Rho. Thus, it appears that the intoxication of macrophage by ExoS could lead to an inhibition of the two major pathways of phagocytosis during *P. aeruginosa* infections.

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*Note Added in Proof*—SptP is a Rac GAP (Fu, Y., and Galan, J. E. (1999) *Nature* 401, 293–297).

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