**Pseudomonas aeruginosa** Exoenzyme S Disrupts Ras-mediated Signal Transduction by Inhibiting Guanine Nucleotide Exchange Factor-catalyzed Nucleotide Exchange*

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**Pseudomonas aeruginosa** exoenzyme S double ADP-ribosylates Ras at Arg\(^{41}\) and Arg\(^{128}\). Since Arg\(^{41}\) is adjacent to the switch 1 region of Ras, ADP-ribosylation could interfere with Ras-mediated signal transduction via several mechanisms, including interaction with Raf, or guanine nucleotide exchange factor-stimulated or intrinsic nucleotide exchange. Initial experiments showed that ADP-ribosylated Ras (ADP-r-Ras) and unmodified Ras (Ras) interacted with Raf with equal efficiencies, indicating that ADP-ribosylation did not interfere with Ras-Raf interactions. While ADP-r-Ras and Ras possessed equivalent intrinsic nucleotide exchange rates, guanine nucleotide exchange factor (Cdc25) stimulated the nucleotide exchange of ADP-r-Ras at a 3-fold slower rate than Ras. ADP-r-Ras did not affect the nucleotide exchange of Ras, indicating that the ADP-ribosylation of Ras was not a dominant negative phenotype. Ras-R41K and ADP-r-Ras R41K possessed similar exchange rates as Ras, indicating that ADP-ribosylation at Arg\(^{41}\) did not inhibit Cdc25-stimulated nucleotide exchange. Consistent with the slower nucleotide exchange rate of ADP-r-Ras as compared with Ras, ADP-r-Ras bound its guanine nucleotide exchange factor (Cdc25) less efficiently than Ras in direct binding experiments. Together, these data indicate that ADP-ribosylation of Ras at Arg\(^{41}\) disrupts Ras-Cdc25 interactions, which inhibits the rate-limiting step in Ras signal transduction, the activation of Ras by its guanine nucleotide exchange factor.

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**Pseudomonas aeruginosa**, a Gram-negative opportunistic pathogen, can cause severe infections in immunocompromised individuals, cystic fibrosis patients, and burn wound victims (1). Exoenzyme S (ExoS), a 49-kDa ADP-ribosyltransferase (2), plays an undefined role in **P. aeruginosa** pathogenesis. ExoS is secreted directly into the eukaryotic cell via the type III secretion mechanism of **P. aeruginosa** (3), a mechanism which requires a direct interaction between the bacterium and the eukaryotic cell. Models of type III translocation predict that the effector protein (ExoS) is translocated directly from the bacterial cytosol into the eukaryotic cell cytoplasm (4). Once in the eukaryotic cytoplasm, ExoS can interact with the FAS protein, a eukaryotic protein of the 14-3-3 family which stimulates the ADP-ribosyltransferase activity of ExoS (5). While ExoS ADP-ribosylates several targets *in vitro* (vimentin (6), IgG\(_3\), and apolipoprotein A1 (7), and several members of the Ras superfamily (8)), only Ras has been identified as an *in vivo* target to date (9).

Ras, a small molecular weight GTP-binding protein, acts as a molecular switch controlling cellular processes ranging from differentiation to proliferation (10). Ras activation involves the exchange of GDP with GTP, while inactivation proceeds through GTP hydrolysis, where bound GTP is hydrolyzed to GDP (11). Growth factors, such as epidermal growth factor, stimulate Ras activation through a signal transduction cascade, involving the recruitment of the Ras guanine nucleotide exchange factor to the membrane where the guanine nucleotide exchange factor catalyzes the exchange of GDP with GTP in Ras (12). Once activated, Ras can interact with several downstream effectors including phosphatidylinositol 3-kinase, Raf, GD35, and Raf (12). Upon interaction with GTP bound Ras, Raf kinase is recruited to the membrane and subsequently activated. Activated Raf initiates a MAP kinase cascade which leads to phosphorylation of transcription factors and changes in gene expression (12).

Recent studies showed that ExoS ADP-ribosylates Ras at two sites *in vitro*: Arg\(^{41}\) and Arg\(^{128}\) (13). Arg\(^{41}\) and Arg\(^{128}\) are located in two distinct structural motifs, α-helix and β-sheet, respectively, and in two distinct regions in the Ras molecule. Arg\(^{41}\) is located adjacent to the switch 1 domain of Ras (14), a domain that changes conformation upon Ras activation. Mutations within the switch 1 domain affect Ras’s ability to interact with the Ras guanine nucleotide exchange factor and its downstream effectors (15). Arg\(^{128}\) is located in a C-terminal α-helix of Ras with no ascribed function. Analysis of a double mutant, Ras-R41K,R128K, showed that ExoS ADP-ribosylated this mutant at Arg\(^{125}\), an Arg that is present within the same α-helix as Arg\(^{128}\). The ability of exoenzyme S to ADP-ribosylate Ras within several structural motifs and at several locations may explain how ExoS can modify several members of the Ras superfamily (13). Intracellular expression of ExoS modulates several cellular functions, including the inhibition of cellular DNA synthesis (16), disruption of Ras-mediated signal transduction in PC12 cells (17), and inhibition of EGF\(^1\) stimulated Raf kinase activity in LNCaP cells.\(^2\) In addition, intracellular expression of the ADP-ribosyltransferase domain of ExoS is cytotoxic to cultured cells (18). In this study, the mechanism by which ExoS disrupts Ras signal transduction was shown to...
occur by inhibiting guanine nucleotide exchange factor-cata-
lyzed nucleotide exchange.

EXPERIMENTAL PROCEDURES

Materials—Mammalian cell culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD). All other chemicals and reagents were purchased from Sigma except where indicated. c-Ha-Ras and FAS were obtained from H. Fu. Plasmids encoding the catalytic domain (amino acid residues 976–1260) of the mammalian GDP/GTP exchange factor of Ras fused to GST, GST-Cdc25Raf25 (Cdc25), was obtained from A. Wittinghofer and plasmids encoding the Ras-binding domain of the Raf kinase (amino acids 1–149) fused to GST, GST-Raf-RBD, was obtained from D. Shalloway. Ras Nucleotide Loading and ADP-ribosylation—Histidine-tagged Ras (Ras) was purified in its nucleotide free form as described previously (13). Ras nucleotide loading and ADP-ribosylation were performed essentially as described (13) with several modifications. For Ras-Raf interaction experiments, nucleotide loading reactions were prepared containing 30 μM Ras, 20 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 200 μM of the respective nucleotide for 30 min at 30 °C. Reactions were stopped by adding MgCl₂ to a final concentration of 20 mM. For analysis of Ras-Cdc25 interactions and Cdc25-catalyzed nucleotide exchange, nucleotide reactions were prepared containing 30 μM Ras, 40 mM Tris-HCl (pH 7.6), 2 mM DTT, and 200 μM of the respective nucleotide for 30 min at 30 °C and reactions were stopped by adding MgCl₂ to a final concentration of 20 mM. The stoichiometry of nucleotide loading of Ras was monitored radioanalytically using 35S-GTPγS. Nucleotide loaded Ras (Ras-GTP or Ras-GDP) was then diluted to a final concentration of 20 μM into ADP-ribosylation reactions containing 100 μM NAD, 0.4 μM ΔN222 ExoS, and 1.6 μM FAS.

Ras Interactions with Immobilized GST-Raf-RBD—Ras-binding domain (Raf-RBD) was purified as described previously (19) with minor modifications. Raf-RBD was not eluted from the GST resin after purification, but instead the resin was washed in 40 mM Tris-HCl, 2 mM DTT, and 10 mM MgCl₂ to remove free radiolabel. Filter bound radioactivity was determined by linear regression analysis, using Sigma Plot (Jandel Scientific).

RESULTS

Previous experiments demonstrated that ExoS double ADP-ribosylated Ras in vitro (17) and during the course of infection in cultured cells (9). In addition, infection of PC12 cells with P. aeruginosa expressing ExoS inhibited Ras-mediated signal transduction (17). Several mechanisms could be responsible for ExoS-mediated inhibition of Ras signal transduction. ADP-ribosylation could inhibit Ras membrane localization, target Ras for degradation, inhibit Ras activation by inhibiting either guanine nucleotide exchange factor-catalyzed or intrinsic nucleotide exchange, or inhibit the interaction between Ras and its downstream effector Raf.

ADP-ribosylation Does Not Interfere with Ras-Raf Interactions—The co-crystal structure of Ras-GTP and Raf predicts that Arg54 of Ras directly contacts Raf (24), suggesting that ADP-ribosylation of Ras at Arg54 may disrupt Ras-Raf interactions. Initial experiments determined the influence of ADP-ribosylation on the binding of Ras to Raf. Ras was first loaded with GTP, and then ADP-ribosylated by ΔN222 ExoS, a catalytic deletion peptide of ExoS containing the ADP-ribosyltransferase domain (25), using conditions which achieved double ADP-ribosylation of Ras (13). Ras-GTP, ADP-r-Ras-GTP, or a mixture of Ras-GTP and ADP-r-Ras-GTP was then added to an immobilized GST fusion protein containing the entire Ras-binding domain of Raf-1 (GST-Raf-RBD). Hybridization analysis of the material that bound to immobilized GST-Raf-RBD showed that similar amounts of Ras-GTP or ADP-r-Ras-GTP bound GST-Raf-RBD (Fig. 1). Analysis of standard amounts of Ras indicated that the ECL signal was proportional to Ras concentration. When either Ras-GTP or ADP-r-Ras-GTP was incubated with the resin alone, no interaction was detected (data not shown). A competition experiment with equivalent amounts of Ras-GTP and ADP-r-Ras-GTP resulted in the binding of equivalent amounts of both proteins to GST-Raf-RBD,
ADP-ribosylation decreased the affinity of Ras for Cdc25. To determine if ADP-r-Ras-GDP interfered with the ability of Cdc25 to catalyze nucleotide exchange of Ras-GDP, the rate of nucleotide exchange of Ras-GDP was measured in the presence of an excess nonradiolabeled ADP-r-Ras-GDP or Ras-GDP. The Cdc25-catalyzed nucleotide exchange rate of Ras-GDP did not differ significantly in the presence of excess Ras-GDP or ADP-r-Ras-GDP (Fig. 2D, Table I), which indicated that ADP-ribosylation of Ras did not result in a dominant negative phenotype.

ADP-ribosylation of Arg is responsible for the inhibition of Guanine Nucleotide Exchange Factor-catalyzed Guanine Nucleotide Exchange of Ras—Exoenzyme S double ADP-ribosylates Ras at Arg and Arg. To determine whether ADP-ribosylation at Arg or ADP-ribosylation at Arg inhibited Cdc25-catalyzed guanine nucleotide exchange of Ras, Cdc25-catalyzed guanine nucleotide exchange rates of ADP-r-Ras-GDP, Ras-GDP, ADP-r-Ras R41K-GDP, and Ras R41K-GDP was measured. The Cdc25-catalyzed guanine nucleotide exchange rate of ADP-r-Ras R41K-GDP did not differ from the rate of exchange for Ras R41K-GDP (Table I), which indicated that ADP-ribosylation at Arg was responsible for the slower rate of nucleotide exchange catalyzed by Cdc25. The exchange rates of Ras-GDP and Ras R41K-GDP differed by about 20% (Table I), indicating that the Arg to Lys mutation at residue 41 had some effect on Cdc25-catalyzed nucleotide exchange. Together, these results indicate that ExoS inhibits Cdc25-catalyzed nucleotide exchange by ADP-ribosylating Ras at Arg.

ADP-ribosylation Inhibits the Binding of Ras to Cdc25—To examine whether ADP-ribosylation decreased the affinity of Ras for Cdc25, the ability of Ras and ADP-r-Ras to bind to immobilized GST-Cdc25 was measured in vitro. Others have demonstrated that nucleotide-free GST-tagged Ras can bind SOS in transfected cell lysates. To examine whether Ras could bind to GST-Cdc25, the binding of Ras to GST-Septorase 4B (control) and GST-Cdc25 (test) was examined. Control experiments showed detectable binding of Ras to GST-Septorase 4B, but the addition of competitor protein, 0.2 mg/ml egg albumin, decreased this nonspecific binding of Ras (data not shown). Subsequent experiments included 0.2 mg/ml egg albumin in the binding assays. Analysis of coprecipitated material indicated that Ras bound to GST-Cdc25 more efficiently than to GST-Septorase 4B (Fig. 3A). Other controls showed that nucleotide-free Ras (Ras-NF) bound more efficiently to GST-Cdc25 than either Ras-GDP or Ras-GTP (Fig. 3A).

Next, the affect of ADP-ribosylation on the ability of nucleotide-free Ras to bind GST-Cdc25 was determined. ADP-r-Ras-NF interacted with GST-Cdc25 less efficiently than Ras-NF, indicating that ADP-ribosylation interferes with the binding of Cdc25 to Ras (Fig. 3A). A competition experiment utilizing equivalent amounts of Ras-NF and ADP-r-Ras-NF resulted in the binding of more Ras-NF than ADP-r-Ras-NF, further indicating that Ras-NF has a higher affinity for Cdc25 than ADP-r-Ras-NF (Fig. 3B). Other experiments showed that the binding of Ras to Cdc25 was dose dependent (data not shown) and that the immunoreactivity was proportional to Ras concentration under the conditions tested (data not shown). We were not able to interpret the binding properties of Ras-R41K since it showed sufficient binding to GST-Septorase 4B in the presence of 0.2 mg/ml egg albumin. This limited the ability to resolve specific binding of Ras-R41K to Cdc25. Together these data showed that Ras-NF interacted with Cdc25 more efficiently than ADP-r-Ras-NF, indicating that ADP-r-Ras has a lower affinity for Cdc25 than unmodified Ras.

ADP-ribosylation of Ras Inhibits GTP Loading of Ras in Vivo—In vitro data indicated that Ras ADP-ribosylation inhibi-
ExoS inhibits GEF-catalyzed nucleotide exchange in Ras.

**Fig. 2.** ADP-ribosylation interferes with Cdc25 catalyzed but not intrinsic Ras nucleotide exchange. 

**A,** the intrinsic rate of nucleotide exchange of unmodified Ras and ADP-ribosylated Ras was measured as described. Reaction mixtures contained 2.5 μM Ras-GDP or ADP-ribosylated Ras-GDP, 500 μM GTPγS, 40 mM Tris (pH 7.6), 2 mM DTT, and 10 mM MgCl₂, and nucleotide exchange was measured over the course of 45 min. Nucleotide exchange rates are reported in Table I. **B,** Cdc25-catalyzed guanine nucleotide exchange of unmodified Ras and ADP-ribosylated Ras was measured as described. Reaction mixtures contained 2.5 μM Ras-GDP or ADP-ribosylated Ras-GDP, 500 μM GTPγS, 33 nM Cdc25, 40 mM Tris (pH 7.6), 2 mM DTT, and 10 mM MgCl₂, and nucleotide exchange was measured over the course of 10 min. **C,** Cdc25-stimulated nucleotide exchange of unmodified Ras and ADP-ribosylated Ras was measured as in B using a concentration of 250 nM Cdc25. **D,** the rate of Cdc25-catalyzed GDP dissociation of unmodified Ras in the presence of either 5 μM Ras-GDP or ADP-ribosylated Ras-GDP, 500 μM GTPγS, 250 nM Cdc25, 40 mM Tris (pH 7.6), 2 mM DTT, and 10 mM MgCl₂ and nucleotide exchange was measured over the course of 10 min.

The current document contains text on the inhibition of guanine nucleotide exchange factor-catalyzed nucleotide exchange by ADP-ribosylation, which prompted an examination of the in vivo effect of ADP-ribosylation on Ras-GTP loading. Growth factors stimulate Ras-mediated signal transduction pathways (19). If Ras ADP-ribosylation inhibited guanine nucleotide exchange factor-catalyzed nucleotide exchange, ExoS should inhibit growth factor-stimulated GTP loading of Ras, but not GTP loading of dominant active Ras mutants (Ras-V12) which do not require growth factor stimulation for GTP loading in vivo. Since Raf-RBD interacts with Ras-GTP at a higher affinity than Ras-GDP, the amount of Ras which bind to immobilized-RBD in cell lysates was used as a measurement of cellular Ras-GTP. Thus, the affect of ExoS on the GTP loading of Ras and Ras-G12V in vivo was measured. Two cell lines were analyzed, one which expressed Ras-G12V (T24) and another which expressed Ras (LNCaP). T24 cells do not require guanine nucleotide exchange factor for Ras-GTP loading, since the Val12 mutation increases the half-life and affinity of Ras for GTP (27) (Fig. 4). Both cell lines were infected with isogenic strains of P. aeruginosa that expressed ExoS (ExoS) or lacked the ExoS gene (∆ExoS). Following infection, LNCaP cells were stimulated with EGF and Ras-GTP was measured as the amount of Ras bound to immobilized RBD in cell lysates. Infection of LNCaP cells with P. aeruginosa expressing ExoS resulted in a decrease in EGF-stimulated GTP loading of Ras, while infection of LNCaP cells with P. aeruginosa ∆ExoS did not decrease GTP loading of Ras (Fig. 4). These results indicate that ExoS inhibits growth factor-stimulated Ras-GTP loading in vivo. In contrast, infection of T24 cells with P. aeruginosa expressing ExoS did not change the amount of GTP loading of Ras relative to uninfected cells, indicating that ExoS does not affect growth factor-independent GTP loading of Ras (Fig. 4). Analysis of a GST fusion containing the R89D mutant of the Ras-binding domain of Raf, a mutant that does not interact with Ras, showed that Ras did not bind to these resins, indicating that Ras does not interact nonspecifically with the GST resin under these conditions (Fig. 4).


**ExoS Inhibits GEF-catalyzed Nucleotide Exchange in Ras**

**TABLE I**

Intrinsic and Cdc25-catalyzed guanine nucleotide exchange rates for wild type and ADP-ribosylated Ras

|                               | Unmodified Ras | ADP-r-Ras |
|--------------------------------|----------------|-----------|
| Intrinsic nucleotide exchange rate | 0.78 ± 0.22 fmol GTP/s | 0.85 ± 0.27 fmol GTP/s |
| Cdc25-catalyzed nucleotide exchange rate | 0.13 ± 0.04 pmol GTP/s | 0.05 ± 0.02 pmol GTP/s |
| Cdc25-catalyzed/intrinsic nucleotide exchange | 167 | 56 |
| Cdc25-catalyzed nucleotide exchange rate of wild type Ras in the presence of excess Ras | -0.042 ± 0.03 | -0.031 ± 0.02 |

a ND, denotes not determined.

b Final concentrations: 33 nM exchange factor and 2.5 μM Ras.

c Final concentrations: 250 nM exchange factor, 5 μM wild type or ADP-r-Ras, and 0.5 μM GDPβS-loaded Ras. In this experiment, the rate of GDP dissociation from Ras was measured.

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**DISCUSSION**

Previous studies determined that exoenzyme S disrupted Ras-mediated signal transduction pathways; infection of PC12 cells with bacteria producing ExoS but not bacteria lacking ExoS disrupted Ras-mediated signal transduction (17) and inhibited EGF-stimulated Raf kinase activity in LNCaP cells. Other studies indicated that ExoS double ADP-ribosylates Ras at Arg41 and Arg128 (13). Arg41 is adjacent to the switch I domain of Ras, a domain that interacts with Raf's downstream effectors and has been shown to be a contact residue in the Rap-Raf co-crystal structure (24).

Since ExoS ADP-ribosylated Ras at one of the contact residues between Rap and Raf (Arg41) (24), it was hypothesized that ADP-ribosylation of Ras disrupted Ras-Raf interactions. In the co-crystal structure of Rap and Raf, residues from the switch 1 domain of Rap and the adjacent β-sheet, which contains Arg41, are involved in hydrogen bonding interactions with Raf (24). However, both ADP-r-Ras-GTP and Ras-GTP interacted with Raf at similar affinities. Thus, the ADP-ribosylation of one of the contact residues did not appreciably affect the affinity of Ras for Raf. One of the electron pairs from the nitrogen atoms in the Arg41 guanidinium side chain of Rap is involved in a hydrogen bonding interaction with Raf. Arginine possesses two nitrogen groups in its side chain and ADP-ribosylation is postulated to occur at one of the two nitrogens in a stereospecific manner (28). Thus, after ADP-ribosylation, the alternate nitrogen in the side chain of arginine still may be able to hydrogen bond with Raf. This may explain how ADP-ribosylation of Ras at Arg41 may not inhibit Ras-Raf interactions. Alternatively, ADP-ribosylation may contribute to the binding of Ras to Raf through other polar or hydrophobic interactions between ADP-ribose and Raf.

The ability of ADP-ribosylation to disrupt the interactions between Ras and Raf in vitro was tested by examining the ability of ADP-ribosylated Val12-Ras to interact with the Ras-binding domain of Raf. Both ADP-ribosylated and unmodified Val12-Ras appear to interact with Raf at similar efficiencies in vitro. If ADP-ribosylation does inhibit Ras-Raf interactions, the change in affinity does not appear to be sufficient to disrupt Ras-Raf interactions in vivo. Others have shown that G12V,R41A Ras binds Raf less efficiently than G12V-Ras in vitro (29). While mutation of Arg41 to alanine affects Ras-Raf interactions.
interactions \textit{in vitro}, ADP-ribosylation of Ras at Arg^{41} does not appear to affect Ras-Raf interactions \textit{in vitro}. This is consistent with the model that the non-ADP-ribosylated nitrogen of the arginine guanidinium group still being able to bind with Raf.

The fact that ExoS disrupted Raf kinase activity, but did not disrupt Ras’s ability to interact with Raf directly indicated that ExoS disrupts Ras signal transduction upstream of the Ras-Raf interaction. The affect of ADP-ribosylation on Ras activation was studied by measuring the effects of ADP-ribosylation on intrinsic Ras guanine nucleotide exchange and Cdc25-stimulated guanine nucleotide exchange \textit{in vitro}. ADP-ribosylated Ras and unmodified Ras possessed similar intrinsic nucleotide exchange rates, indicating that modification of Ras does not affect the ability of Ras to exchange nucleotide. Previous data indicated that ExoS ADP-ribosylated Ras-GTP, Ras-GDP, or Ras-Mg^{2+} (nucleotide free) at similar rates (13). The fact that ExoS can ADP-ribosylate either nucleotide bound form of Ras at similar rates indicates that ADP-ribosylation does not interfere directly with binding of either GTP or GDP to Ras. This is consistent with the observation that ADP-ribosylation did not affect intrinsic nucleotide exchange. Others have also reported that ADP-ribosylation does not interfere with Ras intrinsic nucleotide exchange (8). In addition, the crystal structure of Ras predicts that neither Arg^{41} nor Arg^{128} is involved in nucleotide binding (14), which is also consistent with ADP-ribosylation at Arg^{41} or Arg^{128} not affecting intrinsic nucleotide exchange.

Cdc25 stimulated the guanine nucleotide exchange of ADP-r-Ras at a 3-fold lower rate than Ras. Addition of increasing amounts of Cdc25 to the exchange reaction resulted in stoichiometric nucleotide exchange. The fact that stoichiometric nucleotide exchange can be achieved after manipulating the concentration of the exchange factor indicated that Cdc25 is able to reversibly interact with double ADP-ribosylated Ras, which was consistent with the observation that an excess of ADP-r-Ras did not inhibit the nucleotide exchange of Ras. Together these data indicated that ADP-ribosylated Ras did not inhibit the catalytic mechanism of Cdc25 either by binding more tightly to Cdc25 or binding irreversibly to Cdc25. ADP-r-Ras interacted with Cdc25 less efficiently than Ras in direct binding experiments. Together these data indicate that ExoS inhibits Cdc25 nucleotide exchange by inhibiting Ras-Cdc25 protein-protein interactions. ADP-ribosylation at Arg^{128} did not inhibit Cdc25-stimulated nucleotide exchange, which indicated that it was the ADP-ribosylation of Ras at Arg^{41} that inhibited Ras-Cdc25 interactions. The fact that ADP-ribosylation at Arg^{41} was required to inhibit Cdc25-stimulated nucleotide exchange was consistent with structural data which predicted that Arg^{41} is in a region of close contact in the Ras-SOS crystal structure (30).

\textit{In vitro} data suggested that ExoS inhibited Cdc25-stimulated nucleotide exchange by 3-fold and that the effect was not dominant negative. To examine whether ExoS inhibited Ras signaling during the course of infection in cultured cells, the activation of Ras was measured in two cell lines, one expressing constitutively active Ras and one expressing wild type Ras. Others have demonstrated that constitutively active Ras can become activated independent of growth factor (31). ExoS did not affect the activation of ADP-ribosylated Val^{12}-Ras, consistent with the fact that a guanine nucleotide exchange factor-Ras interaction is not required for activation of Val^{12}-Ras. ExoS did inhibit the activation of wild type ADP-ribosylated Ras. Since ADP-ribosylation of Ras is not dominant negative, ExoS would be required to ADP-ribosylate a large fraction of Ras in the cell in order to inhibit Ras signaling. The fact that ExoS modified a large proportion of Ras during the course of infection is consistent with previous data indicating that ExoS disrupts Ras signaling.

In the current model of Ras activation, SOS exists in a complex with Grb2 in the cytoplasm of the eukaryotic cell. Upon stimulation with a growth factor a receptor tyrosine kinase is activated and the Grb2-SOS complex is recruited to the membrane (12). Recruitment of the Grb2-SOS complex to the membrane increases the local concentration of SOS in the vicinity of Ras. This appears to be the rate-limiting step in Ras activation, since overexpression of Cdc25 in the cytoplasm of the eukaryotic cells increases the concentration of SOS in the cell sufficiently such that Ras activation occurs independent of growth factor (26). Stimulation of cells with growth factor does not result in stoichiometric Ras nucleotide exchange. Both nucleotide binding studies (32) and studies using Ras-Raf interactions as a measure of Ras activation (31) indicate that the increase in Ras-GTP is only several fold after growth factor stimulation. Once Raf is activated, Raf stimulates the MAP kinase kinase (MAPKK1), a dual specificity kinase that subsequently activates p42 MAP kinase (ERK). MAPKK1 phosphorylates ERK by a two-collision distributive mechanism rather than a single collision processive mechanism (33). Therefore, MAPKK1 converts the graded inputs derived through Ras activation into the switch-like outputs which result from ERK activation. ExoS, by inhibiting graded inputs into MAPKK via inhibition of Ras activation, could significantly inhibit the switch like outputs generated by MAPK. The facts that ExoS inhibits the rate-limiting step in Ras activation, that the changes in Ras-GTP are only several fold after growth factor stimulation and that MAP kinase activation is non-linearly dependent on Raf activation is consistent with the model that a 3-fold decrease in Ras-SOS interactions is sufficient to inhibit Ras-mediated signal transduction.

Several toxins have been identified which modify small molecular weight GTP-binding proteins and disrupt the signal transduction of Ras superfamily members. \textit{Clostridium difficile} toxinA and toxinB preferentially modify GDP bound RhoA and glucosylate RhoA at threonine 37, locking Rho in its inactive GDP bound conformation (34, 35). \textit{C. sordelii} LT monoglucosylates GDP-bound Ras at threonine 35, the residue analogous to threonine 37 of Rho (36). This modification inhibits intrinsic nucleotide exchange as well as Ras-Raf interactions. CNF deamidates RhoA at glutamine 63, inhibiting its ability to hydrolyze GTP, resulting in constitutively active RhoA (37). ExoS differs from the toxins described thus far in several respects. First ExoS does not enter eukaryotic cells directly, but is introduced by the contact mediated type III secretion pathway of \textit{P. aeruginosa} (3). Second, ExoS does not have a preference for either GDP or GTP bound Ras (13), whereas the other toxins have a preference for either GTP or GDP bound Ras. Finally, ExoS does not alter an intrinsic function of Ras. Both the modifications of \textit{C. difficile} toxinA and toxinB and \textit{C. sordelii} LT alter the intrinsic rate of nucleotide exchange of Ras while CNF inhibits RhoA intrinsic GTPase activity. Modification of arginine 41 by ExoS is unique in that it does not affect the function of Ras itself and only inhibits the ability of Ras to interact with its activator.

Recent studies have shown ExoS to be a bifunctional cytotoxin. Expression of the carboxyl-terminal domain of ExoS, which comprises the ADP-ribosyltransferase domain, is cytotoxic when expressed in cultured cells (18). Intracellular expression of the amino terminus of ExoS (C234) in eukaryotic cells stimulates actin reorganization, without cytotoxicity, which involves small molecular weight GTPases of the Rho
subfamily. The mechanism by which Cys inhibits Rho signaling is currently under investigation.

Rho and Ras are molecular switches which control numerous cellular processes. Recent signaling studies suggest that there is cross-talk between Rho and Ras family members (39). Ras and Rho proteins also contribute to wound healing processes and tissue regeneration. Recent studies have shown that microinjection of endothelial cells with activated Ras stimulated their motility while microinjection of Ras blocking antibodies inhibited cellular motility that is a component of the wound healing process (40). In addition, hepatocyte growth factor/ scatter factor and epidermal growth factor stimulate cellular motility through the Ras signal transduction pathway (41). Rac and Rho are also involved in motility and tissue regeneration, since dominant negative Rac inhibited the cellular motility stimulated by HGF/SF (41) and inhibition of Rho by either C. difficile toxA and toxB or the C. botulinum C3 transferase inhibited wound healing (42). Inhibition of tissue regeneration and wound healing appear to play a role in the pathogenesis of C. difficile, since treatment of gastrointestinal mucosa with C. difficile toxA and toxB alone inhibit regeneration of the gastric mucosa (42). Thus, ExoS may contribute to the establishment of P. aeruginosa infection by inhibiting wound healing and tissue regeneration by two mechanisms. The amino terminus of ExoS could inhibit Rho function and inhibit wound healing in a manner similar to C. difficile. Alternatively, ExoS could inhibit the cellular motility and angiogenesis required for wound healing by ADP-ribosylating Ras. By inhibiting tissue regeneration and wound healing, ExoS may play a pivotal role in chronic disease by maintaining sites of colonization.

Inhibition of Ras or Rho signaling may also interfere with both innate and acquired immunity. Small molecular weight GTP-binding proteins of the Ras superfamily are also required for cellular processes, such as phagocytosis, as Rho proteins contribute to phagocytosis (38). Since Ras functions upstream of Rho in cellular signaling processes (39), ADP-ribosylation of Ras by ExoS or the inhibition of Rho function by C234 may inhibit phagocytosis of P. aeruginosa by macrophage. Other studies indicate that Ras proteins play critical roles in T cell activation (21). Thus, ExoS may inhibit acquired immunity by inhibiting T-cell activation. Future studies will determine what roles the ADP-ribosyltransferase and the cytoskeleton rearrangement activity of ExoS play in the pathogenesis of P. aeruginosa.

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