Pseudomonas aeruginosa Exoenzyme S ADP-ribosylates Ras at Multiple Sites*

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Anand K. Ganesan‡, Dara W. Frank‡, Ravi P. Misra§, Gudula Schmidt¶, and Joseph T. Barbieri¶

From the Departments of §Microbiology and §Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the ¶Institute for Pharmacology and Toxicology, University of Freiburg, Freiburg D-79104, Federal Republic of Germany

Pseudomonas aeruginosa exoenzyme S (ExoS) ADP-ribosylates Ras to a stoichiometry of ~2 molecules of ADP-ribose incorporated per molecule of Ras, which suggests that ExoS could ADP-ribosylate Ras at more than one arginine residue. SDS-polyacrylamide gel electrophoresis analysis showed that ADP-ribosylated Ras possessed a slower mobility than non-ADP-ribosylated Ras. Analysis of the ADP-ribosylation of in vitro transcribed/translated Ras by ExoS identified two electrophoretically shifted forms of Ras, which was consistent with the ADP-ribosylation of Ras at two distinct arginine residues. Analysis of ADP-ribosylated in vitro transcribed/translated Ras mutants possessing individual Arg-to-Ala substitutions showed that Arg-41 was the preferred site of ADP-ribosylation and that the second ADP-ribosylation event occurred at a slower rate than the ADP-ribosylation at Arg-41, but did not occur at a specific arginine residue. Analysis of bacterially expressed wild-type RasAAX and RasAAXR41K supported the conclusion that Arg-41 was the preferred site of ADP-ribosylation. Arg-41 is located adjacent to the switch 1 region of Ras, which is involved in effector interactions. Introduction of ExoS into eukaryotic cells inhibited Ras-mediated eukaryotic signal transduction since infection of PC-12 cells with an ExoS-producing strain of P. aeruginosa inhibited nerve growth factor-stimulated neurite formation. This is the first demonstration that ExoS disrupts a Ras-mediated signal transduction pathway.

Small molecular weight GTP-binding proteins of the Ras superfamily play an integral role in eukaryotic signal transduction, controlling processes such as cell differentiation and proliferation (1). Several bacterial toxins covalently modify members of the Ras superfamily and alter transduction of intracellular signals. Clostridium botulinum C3 ADP-ribosylates Rho at Asn-41, inhibiting Rho-dependent differentiation (2). Clostridium sordelli lethal toxin glucosylates Ras at Thr-37, inhibiting the epidermal growth factor-stimulated p42/44 mitogen-activated protein kinase signaling pathway (3). Escherichia coli cytolysin neutralizing factor deaminates Gln-63 of Rho, inhibiting intrinsic GTPase activity (4, 5).

Conditions such as cystic fibrosis, leukemia, neutropenia, and burn wounds predispose individuals to infections by P. aeruginosa (6). Numerous factors, including the production of exoenzyme S (ExoS1; 453 amino acids), may contribute to the virulence of P. aeruginosa (7). ExoS, a member of the family of bacterial ADP-ribosyltransferases (8), requires the presence of a eukaryotic protein termed factor-activating exoenzyme S (FAS), a 14-3-3 protein, for expression of ADP-ribosyltransferase activity (9). Unique to most bacterial toxins, ExoS does not have rigid target protein specificity. In vitro, ExoS has been shown to ADP-ribosylate a number of targets, including IgG3 (10), apolipoprotein A-I (10), vimentin (11), and several members of the Ras superfamily (12). Analysis of chemical sensitivity indicated that ExoS ADP-ribosylates these proteins at arginine residues (10, 13).

Initial studies implicated a role for ExoS in the dissemination of P. aeruginosa from burn wounds (14) and in tissue destruction in chronic lung infections (15). However, recent studies have shown that the transposon mutant used in the earlier studies had a disruption in the gene encoding a component of its type III secretion system; thus, this mutant would have pleiotropic effects on the expression of other type III secreted factors in addition to ExoS (16). Two recent studies have implicated a role for the type III secretion apparatus in the delivery of ExoS into eukaryotic cells. Olson and co-workers (17) have shown that incubation of cultured cells with strains of P. aeruginosa expressing ExoS resulted in the ADP-ribosylation of Ras, whereas Forsberg and co-workers (18) have demonstrated that ExoS is cytoxic to cultured cells when delivered by the type III secretion system of the heterologous host Yersinia.

In this study, we examined the biochemical aspects of the ADP-ribosylation of Ras by ExoS. We demonstrate that ExoS ADP-ribosylates c-Ha-Ras at more than one site, with Arg-41 being the preferred site of ADP-ribosylation. In addition, we show that infection of PC-12 cells by a strain of P. aeruginosa that produces ExoS inhibits nerve growth factor (NGF)-stimulated neurite outgrowth, which is the first demonstration that ADP-ribosylation by ExoS disrupts a Ras-mediated signal transduction pathway.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were purchased from the indicated manufacturers: [adenylate phosphate]-5’-PINAD and [3H]S-methyl-adenosine, NEN Life Science Products; Tnt7 quick coupled transcription/translation kit, Promega; Sculptor in vitro mutagenesis kit, Amersham Pharmacia Biotech; bovine serum albumin, Pierce; and DNA oligomers, Operon Technologies, Inc. Recombinant FAS and the pET16b c-Ha-Ras vector were gifts from H. Fu (Emory University). P. aeruginosa 388

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‡ To whom correspondence should be addressed: Dept. of Microbiology, Medical College of Wisconsin, 8701 Watertown Plk. Rd., Milwaukee, WI 53226. Tel.: 414-456-8412; Fax: 414-456-6535; E-mail: toxin@mcw.edu.

The abbreviations used are: ExoS, exoenzyme S; FAS, factor-activating exoenzyme S; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis.
exoenzyme S was purified as described previously (19). *P. aeruginosa* 388 and *P. aeruginosa* 388ΔExoS were cultured as described previously (16).

**Purification of His-tagged Ras Proteins—**Ras proteins were expressed in *E. coli* and purified as described previously (20). Briefly, protease inhibitors (phenylmethylsulfonyl fluoride and aprotonin) were added to the concentrated cell suspensions, which were disrupted with a French press. The lysate was centrifuged (30,000 × g for 20 min) and filtered (0.45-μm filter), and His-tagged Ras proteins were purified by Ni²⁺ affinity chromatography (20). His-tagged Ras proteins were eluted with elution buffer containing 3 mM GTP and 10 mM MgCl₂ diluted to 40% glycerol, and stored at ~20 °C. These proteins were termed RasΔCAAX.

**ADP-ribosylation of RasΔCAAX by ExoS—**Reaction mixtures (25 μl) contained 0.2 mM sodium acetate (pH 6.0), with the indicated amount of [adenylate phosphor-32P]NAD, and RasΔCAAX in the presence and absence of FAS and/or ExoS or ΔN222 (a catalytic deletion peptide of ExoS) (20) at the indicated concentrations. Reactions were performed for 1 h at room temperature and stopped with 0.5 volume of gel loading buffer containing β-mercaptoethanol and boiling. Samples were analyzed by SDS-PAGE followed by autoradiography.

**Construction of M13mp18RasΔCAAX and Site-directed Mutagenesis of RasΔCAAX—**RasΔCAAX was engineered by deletion polymerase chain reaction mutation of pet16b-e-Ha-Ras using primers containing EcoRI and BamHI restriction sites at the 5'- and 3'-ends of the gene, respectively. The resulting polymerase chain reaction product was digested with XbaI and BamHI and ligated into the pET15b vector. The EcoRI-BamHI fragment, encoding the RasΔCAAX gene and the T7 promoter, was then cloned into M13mp18. M13mp18RasΔCAAX was used as the template for site-directed mutagenesis. Mutagenesis was performed essentially following the instructions of Amersham Pharmacia Biotech. DNA primers were constructed to be 10 base pairs complementary to the DNA template flanking the mutation of interest and encoded a Arg-to-Ala substitution (GC to CG). The presence of the mutation was confirmed by sequence analysis. The mutants were designated RasΔAAX, where arginine at residue n of Ras was changed to alanine.

**ADP-ribosylation of in Vitro Transcribed/Translated M13ras by ExoS—**M13ras was expressed as a His-tagged protein in *E. coli*. RasΔCAAX possesses the complete GTP-binding domain and effector functions of Ras, but lacks the four carboxyl-terminal residues, which comprises the farnesylation sequence necessary for membrane targeting. ADP-ribosylation of RasΔCAAX by ExoS showed an absolute requirement for FAS and NAD (Fig. 1). ADP-ribosylated RasΔCAAX possessed a slower electrophoretic mobility as determined by SDS-PAGE relative to non-ADP-ribosylated RasΔCAAX. Other experiments showed that at saturation, ExoS had ADP-ribosylated RasΔCAAX at a stoichiometry of ~2 mol of ADP-ribose bound per mol of RasΔCAAX (Table I). This suggested that ExoS ADP-ribosylates RasΔCAAX at potentially two sites. Similar results were obtained when ExoS ADP-ribosylated full-length e-Ha-Ras (data not shown).

**ADP-ribosylation of in Vitro Transcribed/Translated RasΔCAAX by ExoS—**Biochemical characterization of eukaryotic proteins that had been ADP-ribosylated by ExoS indicated that arginine was the site of ADP-ribosylation (10). Since the presence of two potential sites for ADP-ribosylation within Ras could complicate a biochemical determination of the preferred site for ADP-ribosylation, a molecular approach was used to determine if ExoS ADP-ribosylated Ras at a preferred arginine residue. Briefly, the gene encoding RasΔCAAX was subcloned downstream of a T7 promoter, which was then subcloned into M13mp18 (M13ras). The replicative form of M13ras was isolated and subjected to in vitro transcription/translation using [35S]Met as an intrinsic label. The in vitro transcription/translation mixture, containing [35S]Met-RasΔCAAX, was subjected to ADP-ribosylation by ExoS. ADP-ribosylated [35S]Met-RasΔCAAX was measured as a shift in the electrophoretic mobility of [35S]Met-RasΔCAAX. The results for the ADP-ribosylation of in vitro translated [35S]Met-RasΔCAAX by ExoS are shown in Fig. 2. In vitro translated [35S]Met-RasΔCAAX migrated as a distinct 28-kDa band on SDS-PAGE.

**RESULTS**

**ADP-ribosylation of RasΔCAAX by ExoS—**ExoS ADP-ribosylates several eukaryotic proteins in vitro, including vimentin, IgG, and several members of the Ras superfamily (10, 13). In this study, the ADP-ribosylation of e-Ha-Ras by ExoS was examined. To facilitate expression and purification, a deletion peptide of Ras (termed RasΔCAAX) was expressed as a His-tagged protein in *E. coli*. RasΔCAAX possesses the complete GTP-binding domain and effector functions of Ras, but lacks the four carboxyl-terminal residues, which comprises the farnesylation sequence necessary for membrane targeting. ADP-ribosylation of RasΔCAAX by ExoS showed an absolute requirement for FAS and NAD (Fig. 1). ADP-ribosylated RasΔCAAX possessed a slower electrophoretic mobility as determined by SDS-PAGE relative to non-ADP-ribosylated RasΔCAAX. Other experiments showed that at saturation, ExoS had ADP-ribosylated RasΔCAAX at a stoichiometry of ~2 mol of ADP-ribose bound per mol of RasΔCAAX (Table I). This suggested that ExoS ADP-ribosylates RasΔCAAX at potentially two sites. Similar results were obtained when ExoS ADP-ribosylated full-length e-Ha-Ras (data not shown).

**ADP-ribosylation of in Vitro Transcribed/Translated RasΔCAAX by ExoS—**Biochemical characterization of eukaryotic proteins that had been ADP-ribosylated by ExoS indicated that arginine was the site of ADP-ribosylation (10). Since the presence of two potential sites for ADP-ribosylation within Ras could complicate a biochemical determination of the preferred site for ADP-ribosylation, a molecular approach was used to determine if ExoS ADP-ribosylated Ras at a preferred arginine residue. Briefly, the gene encoding RasΔCAAX was subcloned downstream of a T7 promoter, which was then subcloned into M13mp18 (M13ras). The replicative form of M13ras was isolated and subjected to in vitro transcription/translation using [35S]Met as an intrinsic label. The in vitro transcription/translation mixture, containing [35S]Met-RasΔCAAX, was subjected to ADP-ribosylation by ExoS. ADP-ribosylated [35S]Met-RasΔCAAX was measured as a shift in the electrophoretic mobility of [35S]Met-RasΔCAAX. The results for the ADP-ribosylation of in vitro translated [35S]Met-RasΔCAAX by ExoS are shown in Fig. 2. In vitro translated [35S]Met-RasΔCAAX migrated as a distinct 28-kDa band on SDS-PAGE.
Legend to Fig. 8. Results of two representative experiments are shown. Procedures. Results of two representative experiments are shown. The absolute stoichiometry of ADP-ribosylated Ras was determined by measuring the moles of ADP-ribose incorporated into Ras by subjecting the gel band corresponding to Ras to scintillation counting and dividing this value by the concentration of Ras present in the gel band. The absolute concentration of Ras was determined by subjecting an aliquot of acid-hydrolyzed Ras to amino acid composition analysis.

Proteins were subjected to ADP-ribosylation as described in the legend to Fig. 8. Results of two representative experiments are shown. Proteins were subjected to ADP-ribosylation as described in the legend to Fig. 7. Results of two representative experiments are shown. The absolute stoichiometry of ADP-ribosylated Ras was determined by measuring the moles of ADP-ribose incorporated into Ras by subjecting the gel band corresponding to Ras to scintillation counting and dividing this value by the concentration of Ras present in the gel band. The absolute concentration of Ras was determined by subjecting an aliquot of acid-hydrolyzed Ras to amino acid composition analysis.

Next, the ADP-ribosylation of RasΔCAAX by ExoS was assayed at higher concentrations of ExoS such that multiple sites within Ras would be ADP-ribosylated (Fig. 4). With the exception of RasΔCAAXR41A, ExoS ADP-ribosylated wild-type RasΔCAAX and the Nα mutants, with the appearance of double ADP-ribosylated Ras. In contrast, ExoS ADP-ribosylated RasΔCAAXR41A to only a single electrophoretic mobility shift, which was consistent with the presence of only a single site for ADP-ribosylation. These data also indicated that the second site of ADP-ribosylation was not at a specific arginine residue and that more than one arginine could be ADP-ribosylated at the second site. Since a third ADP-ribosylation event was not observed, it appeared that ADP-ribosylation at the second site excluded a third ADP-ribosylation event.

**TABLE I**

| Protein          | GTP dissociation kinetics (t1/2)a | Stoichiometry of ADP-ribosylationb | Linear velocity of ADP-ribosylationc |
|------------------|-----------------------------------|-----------------------------------|-------------------------------------|
| Wild-type RasΔCAAX | 166 ± 57                          | 2.2 ± 0.3                         | 1.1 ± 0.01                           |
| RasΔCAAX         | 170 ± 69                          | 0.73 ± 0.1                        | 0.22 ± 0.09                          |

a GTP dissociation was measured as described under "Experimental Procedures." Results of two representative experiments are shown.

b Proteins were subjected to ADP-ribosylation as described in the legend to Fig. 8. Results of two representative experiments are shown.

c ADP-ribosylation would be detected. Wild-type [35S]Met-RasCAAX and 11 of the individual Arg-to-Ala mutants of RasCAAX, with the exception of RasCAAXR41A, showed an electrophoretic mobility shift upon ADP-ribosylation by ExoS. In contrast, RasCAAXR41A did not show an electrophoretic mobility shift upon incubation with ExoS. These data suggested that Arg-41 was the preferred site of ADP-ribosylation by ExoS. ADP-ribosylation of in vitro translated [35S]Met-RasCAAX (the wild type and Nα mutants) was also assayed at higher concentrations of ExoS such that multiple sites within Ras would be ADP-ribosylated (Fig. 4). With the exception of RasCAAXR41A, ExoS ADP-ribosylated wild-type RasCAAX and the Nα mutants, with the appearance of double ADP-ribosylated Ras. In contrast, ExoS ADP-ribosylated RasCAAXR41A to only a single electrophoretic mobility shift, which was consistent with the presence of only a single site for ADP-ribosylation. These data also indicated that the second site of ADP-ribosylation was not at a specific arginine residue and that more than one arginine could be ADP-ribosylated at the second site. Since a third ADP-ribosylation event was not observed, it appeared that ADP-ribosylation at the second site excluded a third ADP-ribosylation event.

ADP-ribosylation of Several Arg-41 Mutants of Ras by ExoS—To support the identification of Arg-41 as the preferred site for ADP-ribosylation by ExoS, several more conservative site-directed mutations were engineered at residue 41, lysine (RasCAAXR41K) and glutamine (RasCAAXR41Q). As observed for RasCAAXR41K, low concentrations of ExoS did not ADP-ribosylate either RasCAAXR41K or RasCAAXR41Q. ADP-ribosylation of Several Arg-41 Mutants of Ras by ExoS—To support the identification of Arg-41 as the preferred site for ADP-ribosylation by ExoS, several more conservative site-directed mutations were engineered at residue 41, lysine (RasCAAXR41K) and glutamine (RasCAAXR41Q). As observed for RasCAAXR41K, low concentrations of ExoS did not ADP-ribosylate either RasCAAXR41K or RasCAAXR41Q (Fig. 5). A second positive control, RasCAAXG12V, was ADP-ribosylated by ExoS as measured by the generation of electrophoretic mobility shifted Ras.

ADP-ribosylation of RasΔCAAX and RasΔCAAXR41K by ExoS—To address ExoS-mediated ADP-ribosylation of Ras at Arg-41 in more detail, RasΔCAAX and RasΔCAAXR41K were analyzed as targets for ADP-ribosylation. Bacterially produced RasΔCAAX and RasΔCAAXR41K were expressed and purified to similar levels and reacted with polyclonal antisera to Ras by Western blotting (Fig. 6). To determine whether RasΔCAAX and RasΔCAAXR41K were functional, the GTP dissociation kinetics were measured. Both RasΔCAAX and RasΔCAAXR41K exhibited similar GTP dissociation kinetics (Table I), which indicated that the R41K mutation did not alter the global functional properties of Ras. Others have reported that mutations at Arg-41 did not alter the GTP dissociation kinetics (21).
FIG. 6. Western blotting of wild-type RasΔCAAX and His-
tagged RasΔCAAXR41K. Wild-type RasΔCAAX (WT), RasΔ-
CAAXR41K (K41), and soybean trypsin inhibitor (SBTI, 2 μg) were
subjected to SDS-PAGE. One gel was stained for protein (A), and
proteins in the second gel were transferred to nitrocellulose filters and
incubated with a Ras polyclonal antibody (Upstate Biotechnology, Inc.)
followed by incubation with ¹²⁵I-protein A. The filter was subjected to
autoradiography (B).

CAXR41K by ExoS was studied. At saturation, ExoS ADP-
ribosylated wild-type RasΔCAAX to a stoichiometry of −2 mol of
ADP-ribose incorporated per mol of Ras (Table I), whereas
ExoS ADP-ribosylated RasΔCAAXR41K to a stoichiometry of

0.7 mol of ADP-ribose incorporated per mol of Ras. Examina-
tion of the Coomassie Blue-stained gel and autoradiogram
showed that, relative to non-ADP-ribosylated Ras, ADP-ribo-
sylated RasΔCAAX migrated with an altered electrophoretic
mobility on SDS-PAGE, whereas ADP-ribosylated RasΔCAAXR-
R41K did not have an altered electrophoretic mobility on SDS-
PAGE (Fig. 7). The fact that ADP-ribosylated RasΔCAAXR41K
did not have an altered electrophoretic mobility in this exper-
iment is due to the fact that this experiment was performed using
a 12% polyacrylamide gel, whereas the second ADP-ribosyla-
tion event was observed upon separation on a 15% polyacryl-
amide gel, as seen in Fig. 4. Under linear velocity conditions,
ExoS ADP-ribosylated wild-type RasΔCAAX at a faster velocity
than RasΔCAAXR41K. A representative graph depicting the
velocity of the ADP-ribosylation of wild-type RasΔCAAX and
RasΔCAAXR41K by ExoS is shown in Fig. 8. The average of
three independent experiments indicated that ExoS ADP-ribo-
sylated wild-type RasΔCAAX at 5-fold greater velocity than
RasΔCAAXR41K (Table I).

Infection of PC-12 Cells with P. aeruginosa Expressing ExoS
Inhibits NGF-stimulated Neurite Formation—Arg-41 is located
next to the switch 1 region of Ras (residues 30–39), a domain
that interacts with downstream effectors (Fig. 9) (22). Thus, ADP-ribosylation of Ras at Arg-41 could potentially disrupt eukaryotic signal transduction by inhibiting the interaction of Ras with downstream effectors. The ability of ExoS to disrupt eukaryotic signal transduction was determined by analysis of NGF-stimulated neurite outgrowth in PC-12 cells. NGF-stimulated neurite outgrowth in PC-12 cells follows a Ras-mediated signal transduction pathway, although there are also Ras-independent components to the pathway (23). Since ExoS is secreted from \textit{P. aeruginosa} via a type III secretion pathway (16), ExoS was delivered into PC-12 cells by infection with \textit{P. aeruginosa}. PC-12 cells were incubated alone, with a strain of \textit{P. aeruginosa} that produced ExoS (388), or with a strain of \textit{P. aeruginosa} that was genetically deleted for \textit{exoS} (388D). After 2 h, PC-12 cells were washed to remove unbound bacteria and incubated alone or with NGF. At 24 h post-infection, PC-12 cells were examined by phase microscopy. PC-12 cells that were incubated in the absence of bacteria and NGF showed a rounded morphology, typical of unstimulated cells (Fig. 10). In contrast, PC-12 cells that had been incubated alone or with a strain of \textit{P. aeruginosa} that lacks \textit{exoS} (388DExoS) and then incubated with NGF had undergone a morphological change, where the cells had spread and exhibited neurite outgrowth. PC-12 cells that had been incubated with the ExoS-expressing strain of \textit{P. aeruginosa} followed by incubation with NGF showed a rounded morphology, similar to the morphology of non-infected, non-NGF-stimulated cells. These data indicated that infection of PC-12 cells with an ExoS-producing strain of \textit{P. aeruginosa} inhibited NGF-stimulated signal transduction leading to neurite outgrowth. In other experiments, we observed that transient expression of ExoS from a cytomegalovirus promoter also inhibited NGF-mediated neurite outgrowth in PC-12 cells (2). Together, these data indicated that inhibition of NGF-mediated neurite outgrowth was due to the ADP-ribosyltransferase activity of ExoS and not a general cytotoxicity of ExoS or \textit{P. aeruginosa} for PC-12 cells.

**DISCUSSION**

Earlier studies predicted that ExoS ADP-ribosylates eukaryotic proteins at arginine residues (10, 12). We initially pursued a biochemical analysis of the site of ADP-ribosylated Ras. However, we found that, during proteolysis, a considerable amount of radiolabel was released as ADP-ribose and that the radiolabel that remained peptide-associated was present in several peptides (2). This, in addition to the observation that ExoS ADP-ribosylated Ras to a stoichiometry of 2 (Table I), prompted a molecular approach for the characterization of the site of ADP-ribosylation. The subsequent determination that ExoS ADP-ribosylates Ras at multiple sites is consistent with our inability to identify a specific arginine as the preferred site of ADP-ribosylation by biochemical approaches. Characterization of \textit{in vitro} transcribed/translated forms of Ras that each possessed one of 12 individual Arg-to-Ala substitutions identified Arg-41 as the preferred site for ADP-ribosylation since the Arg-41 mutants of Ras were not ADP-ribosylated by low concentrations of ExoS. Also, examination of the velocities of ADP-ribosylation of Ras by ExoS showed that Arg-41 was essentially completely ADP-ribosylated prior to the appearance of the second ADP-ribosylation event, which was consistent with Arg-41 being the high affinity site of ADP-ribosylation within Ras (3).

Analysis of the single Arg mutants of Ras did not identify a specific Arg as the second site for ADP-ribosylation since all of the single arginine residues could constitute the second site of ADP-ribosylation within Ras. Since a third ADP-ribosylation event was not observed, it appeared that the ADP-ribosylation at one arginine residue excluded a third ADP-ribosylation event. This suggests that the arginines that are targeted for the second ADP-ribosylation event are located in close proximity in the three-dimensional structure of Ras. These data also showed that the ADP-ribosylation at Arg-41 and that at the second arginine were independent events and not the result of sequen-

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tial, ordered ADP-ribosylation reactions since the Arg-41 mutants could be ADP-ribosylated at the second arginine residue. There is precedence for the ADP-ribosylation of proteins at two sites. Aktories and co-workers (24) recently showed that an endogenous eukaryotic ADP-ribosyltransferase modifies actin at Arg-95 and Arg-372.

The ability of ExoS to ADP-ribose Ras at multiple arginine residues assists in addressing an earlier report by Coburn et al. (12) that described the ADP-ribosylation of several members of the Ras superfamily by ExoS and the subsequent prediction that ExoS ADP-ribose Ras at Arg-123 (13). Our data could be consistent with Arg-123 being one of the secondary sites of ADP-riboseylation within Ras.

Examination of the x-ray crystallographic structure of Ras (25) predicts that Arg-41 lies adjacent to the switch 1 domain, which has been shown to function in the interactions with downstream effectors, particularly Raf (22). Alignment of the primary amino acid sequences of members of the Ras superfamily of small molecular weight GTP-binding proteins showed that Arg-41 is not a highly conserved residue. Thus, it appears that ExoS can potentially ADP-ribose the Arg-41 homologue of Ras, Rat, and Rap, but that members of the Rab or Rho family will not be targeted for ADP-riboseylation by ExoS at the Arg-41 homologue. However, the observation that ExoS may ADP-ribose Ras at a second arginine residue poses the possibility that other members of the Ras superfamily that lack the Arg-41 homologue, including Rho and Rab, may be targeted for ADP-riboseylation by ExoS at an arginine residue that corresponds to the second site of ADP-riboseylation within Ras. The ADP-riboseylation of other Ras superfamily members by ExoS is currently under investigation.

Neurite outgrowth in PC-12 cells is a multistep process that is initiated by NGF binding to the Trk membrane tyrosine kinase. NGF binding stimulates Trk kinase activity, which results in the phosphorylation of several effector molecules that activate Ras. This pathway has both Ras-dependent and independent components. Several studies have suggested that Ras is a dominant element in the transmission of NGF signals from Trk, including microinjection of antibodies to Ras, which interferes with the signaling pathway (26), or introduction of dominant inhibitory Ras alleles into PC-12 cells, which inhibits NGF-induced neurite outgrowth. In addition, transfection of constitutively active Ras, in the absence of NGF stimulation, is sufficient to induce neurite outgrowth (26). The Ras switch 1 domain is required for this signal transduction process since point mutations within the switch 1 regions of constitutively active RasV12 interfere with neurite outgrowth (21). The fact that ExoS preferentially modifies Ras at a region adjacent to the switch 1 domain and that infection of PC-12 cells with strains of P. aeruginosa expressing ExoS results in an inhibition of neurite outgrowth in PC-12 cells is consistent with the model that ExoS inhibits Ras-mediated signal transduction in vivo. However, since ExoS has the potential to modify a wide variety of targets in vitro, it is conceivable that ExoS can disrupt PC-12 cell signal transduction by the ADP-riboseylation of components of this signal transduction pathway. Future studies will focus on whether ExoS disrupts PC-12 cell signal transduction via modification of other components involved in PC-12 cell signal transduction.

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