Auto-ADP-ribosylation of *Pseudomonas aeruginosa* ExoS

P. aeruginosa Exoenzyme S (ExoS) is a bifunctional type-III cytotoxin. The N terminus possesses a Rho GTPase-activating protein (GAP) activity, whereas the C terminus comprises an ADP-ribosyltransferase domain. We investigated whether the ADP-ribosyltransferase activity of ExoS influences its GAP activity. Although the ADP-ribosyltransferase activity of ExoS is dependent upon FAS, a 14-3-3 family protein, factor-activating ExoS (FAS) had no influence on the activity of the GAP domain of ExoS (ExoS-GAP). In the presence of NAD and FAS, the GAP activity of full-length ExoS was reduced about 10-fold, whereas NAD and FAS did not affect the activity of the ExoS-GAP fragment. Using[^32P]NAD, ExoS-GAP was identified as a substrate of the ADP-ribosyltransferase activity of ExoS. Site-directed mutagenesis revealed that auto-ADP-ribosylation of Arg-146 of ExoS was crucial for inhibition of GAP activity in *vitro*. To reveal the auto-ADP-ribosylation of ExoS in intact cells, tetanolysin was used to produce pores in the plasma membrane of Chinese hamster ovary (CHO) cells to allow the intracellular entry of[^32P]NAD, the substrate for ADP-ribosylation. After a 3-h infection of CHO cells with *Pseudomonas aeruginosa*, proteins of 50 and 25 kDa were preferentially ADP-ribosylated. The 50-kDa protein was determined to be auto-ADP-ribosylated ExoS, whereas the 25-kDa protein appeared to represent a group of proteins that included Ras.

ExoS is a bifunctional cytotoxin. The N terminus possesses Rho GTPase-activating protein (GAP) activity (3), and the C terminus is an ADP-ribosyltransferase (4). *In vitro*, ExoS is a GAP for the Rho GTPases Rho, Rac, and Cdc42 (3). Consistent with the functions of Rho GTPases as regulators of the actin cytoskeleton, transfection of the N terminus of ExoS into cultured cells induced actin redistribution (5, 6). Recent crystallographic studies revealed that the GAP domain of ExoS comprises seven α-helices linked by two loop regions (7). The Rho GAP domain of ExoS is similar to, yet distinct from, the eukaryotic Rho GAPs.

The C terminus of ExoS is a 14-3-3-dependent ADP-ribosyltransferase (8). Transfection of the C terminus into cultured cells results in cell death (9). Although cell death requires expression of ADP-ribosyltransferase activity, the mechanism responsible for eliciting cytotoxicity has not been established. *In vitro*, ExoS ADP-ribosylates numerous proteins (10), including members of the Ras subfamily of monomeric GTPases. ExoS ADP-ribosylates multiple arginine residues in Ras and Rap, with Arg-41 the preferred site of ADP-ribosylation (11). ADP-ribosylation of Arg-41 inhibits binding of Ras GTPases to their specific guanine nucleotide-exchange factor (4).

Here we report that ExoS is auto-ADP-ribosylated *in vitro*, resulting in a reduction of its GAP activity. Utilizing tetanolysin as a pore-forming toxin to allow the intracellular entry of[^32P]NAD, ExoS was observed to ADP-ribosylate various eukaryotic substrates, including Ras. Moreover, ExoS was auto-ADP-ribosylated in CHO cells, suggesting an intramolecular regulation of the functions of ExoS in intact cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were purchased: nickel-nitroliotropic acid-Agarose (Qiagen), glutathione-Sepharose 4B beads (Amersham Biosciences, Inc.), tetanolysin and Clostridium botulinum Exoenzyme C3 (List Biologicales),[^32P]NAD (ICN), His-probe and a BCA protein assay kit (Pierce), mouse α-Hras antibody (Transduction Laboratories), rat α-pun Ras antibody Y13-259 (Sigma), mouse α-GFP antibody (BAbCO), and rabbit α-HA antibody (Covance). *P. aeruginosa* PA103 ΔexoU, exoT::Tet(ρUCP), which does not express the four known type III effectors (ExoS, ExoT, ExoU, ExoY), and *P. aeruginosa* PA103ΔexoU, exoT::Tet(ρUCPExoS), which expresses only ExoS, were obtained from Daru Frank. Human α-ExoS antiserum has been described previously (12). Cell culture reagents were purchased from Amersham Biosciences, Inc.

**Purification of Proteins and Fragments**—Recombinant proteins were produced in *Escherichia coli* BL21 cells and purified by Ni²⁺ affinity chromatography (ExoS fl, ExoS-terrafase, and FAS (Haian Fu, Emory University) or affinity chromatography with glutathione-Sepharose (ExoS-GAP). Overnight cultures were diluted 1:10 with fresh LB medium. After 2 h at 30 °C, isopropyl-β-D-thiogalactopyranoside (0.2 mM, final concentration) was added. After an additional 2-h incubation, cells were harvested and lysed by sonication in lysis-buffer (20 mM Tris/HCl, pH 7.4, 10 mM NaCl, 5 mM MgCl₂, and 1% Triton X-100). His-tagged ExoS was extracted from the lysate with 1 ml of nickel-
nitrilotriacetic acid-Agarose, which was washed three times in 10% glycerol in PBS, pH 6.0. Proteins were eluted from the resin in 0.5 M imidazole, 10% glycerol in PBS, pH 6.0, and stored at 20 °C. GST-ExoS was purified from the lysate by incubation with 1 ml of glutathione-Sepharose 4B beads. Beads were washed twice in 20 ml Tris/HC1, pH 7.4, 10 mM NaCl, and 150 mM NaCl and 50 mM Tris/HC1, pH 7.5 at 4 °C before ExoS was released by thrombin cleavage (200 μM/ml thrombin, 150 mM NaCl, 50 mM triethanolamine/HCl, pH 7.5, and 2.5 mM CaCl2, for 45 min at room temperature). Thrombin was removed by incubation with benzamidine-Sepharose, and the protein was stored in aliquots at 20 °C.

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 previously described (13).

Rho GTPase Assay—Recombinant Rho proteins (2 μM, final concentration) were loaded with [γ-32P]GTP (for 5 min at 37 °C in 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM dithiothreitol, MgCl2 (12 mM, final concentration) and unlabeled GTP (2 mM, final concentration) were added alone to stimulate intrinsic GTPase activity or with Exo-S-GAP at 37 °C. GTPase hydrolysis was determined by a filter-binding assay.

ADP-ribosylation of Ras, ExoS, and the Exo-S-GAP Domain—Ras (2 μM) or ExoS-GAP (2 μM) was incubated for 30 min at 29 °C with the indicated concentrations of ExoS-transferase (ExoS full-length (2 μM) was incubated without isolated transferase domain) in a reaction containing 0.2 mM sodium acetate (pH 6.0), 2 mM MgCl2, 100 μM [32P]NAD (specific activity of 25 Ci/μmol of NAD) and 1 μM FAS. Reactions were stopped with gel-loading buffer and boiling. Samples were analyzed by SDS-PAGE followed by autoradiography.

Microinjection of Embryonic Dorsal Cells—For microinjection, EBL cells were seeded subconfluently onto glass coverslips (CELLocate, Eppendorf) and cultivated for 24 h in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in humidified CO2 at 37 °C. Purified ExoS1-234 was incubated for 30 min at 29 °C with the transferase domain (ExoS-GAP: transferase domain = 1:10) or with buffer in the presence of NAD (1 mM) and FAS (1 μM).

ADP-ribosylated and unmodified ExoS-GAP (both 800 μg/ml, in 50 mM Tris-HCl (pH 7.4)) was microinjected into EBL cells with an Eppendorf 5242 microinjector. As negative control ExoS146K (mutant with no GAP activity, 800 μg/ml, in 50 mM Tris-HCl (pH 7.4)) was injected. Photographs were taken 90 min after injection.

Culture and Transfection of Chinese Hamster Ovary-K1 (CHO) Cells—CHO cells were grown under 5% CO2 at 37 °C in F12 medium containing 10% FCS. Cells were transfected with pUCP-ExoS (pUCP) or P. aeruginosa PA103 ΔexoU, ΔexoT, ΔexoC (pUCP-ExoS) was added to CHO cells followed by incubation under 5% CO2 at 37 °C.

CHO Cell Permeabilization and Detection of ADP-ribosylation Activity—P. aeruginosa-infected CHO cells were permeabilized with tetanolysin (List Biologicals, CA) by a procedure adapted from Ahnert-Hilger et al. (14). Confluent CHO cells (85-mm dishes) were washed with 10 ml of PBS and incubated with 6 ml of serum-free F12 medium, containing 0.1% NaHCO3, P. aeruginosa PA103 ΔexoU, ΔexoT, ΔexoC (pUCP-ExoS) was added to CHO cells followed by incubation under 5% CO2 at 37 °C for 2, 3, or 4 h. Medium was removed, and CHO cells were washed with 10 ml of PBS before treatment with tetanolysin, as described in the next section. In 12-well plates, CHO cells were treated in the same way except that cells were washed with 1 ml of PBS and incubated in 0.7 ml of serum-free medium during infection.

CHO Cell Permeabilization and Detection of ADP-ribosylation Activity—P. aeruginosa-infected CHO cells were permeabilized with tetanolysin (List Biologicals, CA) by a procedure adapted from Ahnert-Hilger et al. (14). Cultured CHO cells (85-mm dishes) were washed with 10 ml of PBS and incubated with 6 ml of ice-cold HGI buffer (20 mM PIPES, 2 mM NaATP, 4.8 mM MgCl2, 150 mM potassium glutamate, 2 mM EGTA, and KOH to pH 7.0) containing 1 mM dithiothreitol. Cells were incubated for 10 min at 4 °C, without or with 2.5 μM tetanolysin, and washed with ice-cold HGI buffer, before 6 ml of HGI buffer containing 20 μM [32P]NAD (6 μCi/l) was added. After 25 min at 37 °C, the CHO cells were harvested in 0.5 ml of HB2 buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, and 0.5 mM EDTA). CHO cells were broken by passage (14 times) through a 25-gauge syringe, unbroken cells and nuclei were removed by centrifugation in a microcentrifuge at 2500 rpm for 5 min. Post-nuclear supernatants were centrifuged (68,000 × g in a TLA 100.3 Beckman rotor for 30 min) to obtain supernatant (cytosol) and pellet (membrane) fractions. Pellets were suspended in a volume of HB2 containing 1% Triton equal to that of the supernatant. CHO cells in 12-well plates were permeabilized with 0.4 μg of tetanolysin in 0.6 ml of HGI buffer containing 1 mM dithiothreitol. Cells were harvested directly into 125 ml of SDS-PAGE sample buffer with β-mercaptoethanol and boiled. Proteins were separated by SDS-PAGE and transferred to PVDF, which was exposed to x-ray film or for Western blot experiments subjected to ECL (Pierce) as described by the manufacturer. Primary antibody dilutions were 1/1000 (α-ExoS), 1/2000 (α-GFP), or 1/5000 (His probe).

CHO Cell Toxicity Assays—Trypan blue permeability was assayed as previously described (5). Briefly, 0.4% Trypan Blue stain (Invitrogen) was added to CHO cells. After 5 min, stain was removed, and cells were washed twice with PBS and inspected by light microscopy.

RESULTS

FAS Is Required for ADP-ribosyltransferase of ExoS and Does Not Inhibit Rho-GAP Activity—ExoS ADP-ribosylates members of the Ras family at multiple sites, including Arg-41 and Arg-128, which disrupt Ras-mediated signal transduction (11). ExoS requires Factor-Activating Exoenzyme S (FAS), a widely distributed member of the 14-3-3 protein family, to catalyze ADP-ribosylation (15, 16). As shown in Fig. 1, ExoS transferase (residues 232–453) ADP-ribosylates Ras and Rap only in the presence of NAD and FAS.

The N terminus of ExoS stimulates GAP hydrolysis by Rho GTPases (3). To assess the influence of FAS on the Rho-GAP activity of ExoS, [γ-32P]GTP-bound RhoA was incubated with ExoS-GAP (residues 1–231) or full-length ExoS without and with FAS and GTPase activity was measured. As shown in Fig. 2 (top), the presence of FAS did not enhance GTP hydrolysis by Rho proteins stimulated by the ExoS-GAP fragment. Similar results were obtained for ExoS-GAP-stimulated GAP hydrolysis by Rac and Cdc42 (not shown). Consistent with these findings, the GAP activity of full-length ExoS was not altered by FAS (Fig. 2, bottom). Also an increasing amount of FAS (up to 10 μM) had no influence on the GAP activity of ExoS. Moreover, FAS is not needed for Rho-GAP activity (not shown).

Auto-ADP-ribosylation of ExoS-GAP Blocks GAP Activity—To test if the GAP domain of ExoS is ADP-ribosylated, ExoS-GAP was incubated with different concentrations of the transferase domain (amino acids 232–453) and [32P]NAD with FAS, and the extent of labeling was analyzed. As shown in Fig. 3, the transferase domain catalyzed the ADP-ribosylation of the GAP domain in a concentration-dependent manner. ADP-ribosylation was detectable after incubation of 1 nM transferase with 2 μM GAP domain for 30 min at 30 °C. Modification was maximal with 50 nm transferase, indicating a catalytic action of the transferase.
The effect of ADP-ribosylation of ExoS on its ability to stimulate RhoA GTPase activity was determined (Fig. 4). The GAP domain of ExoS that had been pretreated with NAD and FAS retained its RhoA GAP activity. In contrast, full-length ExoS that had been pretreated with both FAS and NAD had reduced RhoA GAP activity. Because FAS and NAD are essential for ADP-ribosyltransferase, it appeared that the full-length toxin had auto-ADP-ribosylated the GAP domain, which resulted in the inactivation of GAP activity.

Arginine 146 of ExoS Is a Preferred Site of Modification—Because Arg-146 is essential for the GAP activity of ExoS (3), we asked if Arg-146 was ADP-ribosylated. When Arg-146 of ExoS was replaced by Lys, the auto-ADP-ribosylation of ExoS was significantly reduced (Fig. 5A), suggesting preferential, but not exclusive, modification of Arg-146 of ExoS. Quantification of the total amount of radiolabel incorporated under optimal conditions showed that the GAP domain was ADP-ribosylated by about 76 ± 15%. By contrast, up to 37 ± 3% of the mutant R146K was modified under the same conditions. Furthermore, we studied the incorporation of radioactively labeled ADP-ribose into full-length ExoS with the time (Fig. 5B). We found a maximal ADP-ribosylation of full-length ExoS after 15–30 min of incubation. Quantification of the data obtained after 15 min resulted in incorporation of 45.5 ± 4.3 pmol radiolabel into 50 pmol of full-length ExoS. Samples modified under the same conditions were used for the analysis of GAP activity.

**ADP-ribosylation of ExoS-GAP Blocks GAP Activity in Vivo—**To analyze the effects of the ADP-ribosylation of the GAP domain of ExoS, we incubated purified ExoS1-234 with the transferase domain or with buffer in the presence of NAD and FAS and afterward microinjected the proteins into EBL (embryonic bovine lung) cells. We studied the morphology of...
the cells after 90 min. Microinjection of ExoS-1-234 led to retraction of cells (Fig. 6A). By contrast, after injection of the GAP domain, which was preincubated with the transferase in the presence of NAD and FAS, no retraction of the cells was detected, indicating that ADP-ribosylation blocked the GAP activity of the protein (Fig. 6B). As a negative control, we injected ExoS R146K, which has no GAP activity. Cells injected with the GAP-deficient ExoS mutant R146K showed no retraction (Fig. 6C).

Tetanolysin Permits the Entry of [32P]NAD for Detection of Intracellular Protein ADP-ribosylation—To assess the ADP-ribosylation of ExoS in cultured CHO cells and to test whether auto-ADP-ribosylation occurs in vivo, tetanolysin was used to introduce pores in the plasma membrane and permit entry of [32P]NAD. The protocol used for pore formation in CHO cell plasma membranes essentially followed the procedure described by Ahnert-Hilger et al. (14). C3 toxin, which ADP-ribosylates intracellular Rho (17), was used to test the permeability of the tetanolysin pores to NAD. Without tetanolysin treatment, C3 toxin did not incorporate radiolabel from [32P]NAD into intracellular proteins (Fig. 7, lane 1). After tetanolysin treatment, C3 toxin catalyzed the incorporation of radiolabel from [32P]NAD into a single protein (Fig. 7, lanes 2 and 3) of $\sim$25 kDa, the predicted molecular mass of Rho, which indicated that tetanolysin produced pores in the cell membrane that allowed the entry of C3 and [32P]NAD. This system could, therefore, be used to evaluate the intracellular ADP-ribosylation of proteins catalyzed by P. aeruginosa (type-III)-delivered ExoS.

Intracellular ADP-ribosylation by ExoS during P. aeruginosa Infection—CHO cells were infected with an ExoS-producing strain of P. aeruginosa, PA103 ΔexoU, exoT::Tc (pUCP-ExoS),...
or an isogenic strain of *P. aeruginosa* transformed with the vector control (pUCP), for 2, 3, or 4 h. Infected cells were washed to remove unbound *P. aeruginosa*, treated with tetanolysin, and incubated with $^{32}$P]NAD. After a 2-h infection with ExoS-producing *P. aeruginosa*, radiolabeled proteins were not detected in cell lysate (data not shown). After a 3-h infection, CHO cell lysates contained several radiolabeled proteins, the appearance of which was dependent on tetanolysin treatment (compare Fig. 8, 3 h, – and 3 h, +). The two predominant radiolabeled proteins had apparent molecular masses of 25 and 50 kDa. Tetanolysin dependence of this radiolabeling indicated that the integrity of the CHO cell plasma membrane had been retained. Most radiolabeled proteins were in the membrane fraction (Fig. 8), although about 20% of the radiolabeled 50-kDa protein was in the cytosol.

After a 4-h infection with ExoS-producing *P. aeruginosa*, cell lysates contained more radiolabeled protein than after the 3-h infection and the ratio of $^{32}$P in the 50-kDa protein to that in the 25-kDa protein increased, from 0.63 (± 0.27) at a 3-h infection to 1.70 (± 0.10) at a 4-h infection (Fig. 8), suggesting accumulation of the 50-kDa protein during the infection. After a 4-h infection, the incorporation of radiolabeled protein was no longer dependent on tetanolysin (compare Fig. 8, 4 h, – and 4 h, +), indicating increased permeability of the plasma membrane in infected cells with ExoS-producing *P. aeruginosa*, which was confirmed by demonstration of permeability toward Trypan blue (data not shown). Radiolabeling of proteins in *P. aeruginosa*-infected cells was ExoS-dependent, because a 4-h infection with a vector-containing strain of *P. aeruginosa*, *P. aeruginosa* (PA103 ΔexoU, exoT::Tc (pUCP)), did not result in the radiolabeling of proteins after tetanolysin treatment and incubation with $^{32}$P]NAD (Fig. 8, 4 h*, +). A 4-h infection with the vector-containing strain did not result in increased permeability toward Trypan blue (data not shown).

Endogenous Ras Is an Early Target of ExoS during Infection—α-Ras antibody immunoprecipitated a radiolabeled protein that co-migrated with the 25-kDa, radiolabeled protein (Fig. 9A, lane 4). Experiments were performed to determine the efficiency of immunoprecipitation to determine the amount of Ras present in the radiolabeled 25-kDa protein. His$_{6}$-HRas-transfected CHO cells and control CHO cells that had been infected with ExoS-producing *P. aeruginosa* were treated with tetanolysin and $^{32}$P]NAD, and cell lysates were prepared. Immunoprecipitation of His$_{6}$-HRas from cell lysates yielded a major radiolabeled band unique to His$_{6}$-HRas-transfected cells (Fig. 9A, lane 6), and a lower molecular weight band. The lower molecular weight band appears to be a degradation product of His$_{6}$Ras, because it is reactive in His-probe Western blots (data not shown). In immunoprecipitations, the intensity of radiolabeled endogenous Ras is one-fifth His$_{6}$Ras, whereas in lysates, the intensity of radiolabel between 25 and 30 kDa is only 1.3 times greater in His$_{6}$-HRas-transfected CHO cells (Fig. 9B). This indicates that Ras is a minor component of the radiolabeled 25-kDa proteins.

Auto-ADP-ribosylation of ExoS during Infection of CHO Cells by *P. aeruginosa*—The 50-kDa radiolabeled protein in lysates from CHO cells infected with ExoS-producing strains of *P. aeruginosa* co-migrated with authentic ExoS on SDS-PAGE (Fig. 9B), and the amount of radiolabeled 50-kDa protein increased with time of infection. Immunoreaction of the 50-kDa radiolabeled protein with antibody against α-ExoS confirmed its identification (Fig. 10, top). The lysates also contained a radiolabeled protein with an apparent molecular mass of 43 kDa that reacted with α-ExoS antibody after longer exposure of x-ray film (data not shown), which appeared to be a processed form of ExoS. In lysates of CHO cells infected with *P. aeruginosa* containing control vector, a radiolabeled α-ExoS reactive protein was not detected (Fig. 10, top, Bck).

**DISCUSSION**

*Pseudomonas aeruginosa* ExoS is a bifunctional toxin containing N-terminal Rho-GAP and C-terminal ADP-ribosyltransferase domains. The ADP-ribosyltransferase activity depends strictly on the 14-3-3 protein FAS. Pretreatment of full-length ExoS with either FAS or NAD did not alter Rho-GAP activity; however, pretreatment with both NAD and FAS resulted in an inhibition of Rho-GAP activity, suggesting that auto-ADP-ribosylation interfered with Rho GTPase stimulation. The activity of the GAP domain (ExoS1–234) was also inhibited in the presence of the transferase domain (ExoS232–453) with NAD and FAS. In addition, auto-ADP-ribosylation of the Rho-GAP domain reduced its activity by about one order of magnitude.

In *vitro*, the substrate specificity of ExoS ADP-ribosyltransferase is rather broad, and ExoS appears to ADP-ribosylate...
more than one arginine residue in its in vivo target Ras (11). We sought to identify the acceptor amino acids in the GAP domain of ExoS that were modified by the C-terminal transferase domain. Previous mutational studies indicate that Arg-146 is important for expression of Rho-GAP activity (3). Our studies reveal that Arg-146 (one of 11 in Rho-GAP domain of ExoS) is a site of preferential modification. Therefore, auto-ADP-ribosylation of Arg-146 by ExoS transferase blocks its GAP activity. Recently, it was shown that Arg-146 of ExoS is also essential for its cytotoxic effects (6). In line with this notion is our finding that antibodies to identify the modified protein are available. Auto-ADP-ribosylation of ExoS 12087 was not defined. The nature of these posttranslational modifications was not defined.

Analysis of the crystal structure of the ExoS Rac complex corroborates the importance of Arg-146 (7). Arg-146 stabilizes the transition state of GTP hydrolysis by Rho by positioning the interacting partners in the nucleotide hydrolysis reaction. A similar arginine finger is present in all known Rho GAPs. In the absence of Rho, Arg-146 is exposed on the surface of the ExoS molecule and is accessible to the transferase (18).

To permit the identification of intracellular substrates for ADP-ribosylation by ExoS, tetanolysin was used for pore formation in target cells to permit entry of [³²P]NAD. In a frequently used alternative approach, intact cells are incubated with or without the ADP-ribosyltransferase and, thereafter, proteins in the cell lysate are subjected to in vitro ADP-ribosylation reactions (19). Prior intracellular ADP-ribosylation is evidenced by the absence of labeling of a target protein in vitro. This approach has been used to provide a global assessment of in vivo ADP-ribosylation by several toxins, including cholera toxin and pertussis toxin. A limitation of this approach is that it is optimal only when both in vivo and in vitro ADP-ribosylation can be carried to completion. Utilization of this approach with ExoS failed to identify in vivo substrates, because labeling to completion in vitro resulted in the modification of hundreds of proteins (data not shown). Another approach used to detect in vivo ADP-ribosylation is the identification of a shift in the apparent molecular weight of proteins from cells treated with the ADP-ribosyltransferase. This method was used to demonstrate in vivo modification of Ras by ExoS (20) but is not applicable to global assessment of modified substrates. Furthermore, this approach requires that ADP-ribosylation shifts the electrophoretic mobility of a protein and that antibodies to identify the modified protein are available. ADP-ribosylation of EF-2 by diphtheria toxin did not shift the apparent molecular weight (21). Moreover, molecular weight shift is an indirect measure of ADP-ribosylation. Shifted forms of wild-type ExoS introduced into cultured cells by Yersinia were observed and were absent when the ExoS was catalytically inactive (22). The nature of these posttranslational modifications was not defined.

To assess in vivo ADP-ribosylation, a tetanolysin-based approach was developed. Tetanolysin is a 55-kDa member of the streptolysin O family of cholesterol-dependent pore-forming toxins. Tetanolysin pores, which permit diffusion of small proteins and molecules, have been used frequently, e.g. in studies of cAMP- and Ca²⁺-induced effects in T-cell signal transduction (23, 24). Exoenzyme C3, which in cells specifically ADP-ribosylates only Rho (25), was employed to establish proof-of-principle for the detection of intracellular ADP-ribosylation where addition of C3 and [³²P]NAD resulted in incorporation of radiolabel into a single 25-kDa protein. Thus, an assay with pore-forming proteins can be useful for identifying targets of ADP-ribosylation in vivo, because it permits observation of the entire population of intracellular proteins.

Tetanolysin-mediated entry of [³²P]NAD is practical for in-
ADP-ribosyltransferase activity. The tetanolysin-independence of NAD diffusion indicated the loss of membrane integrity after 4 h of infection, a finding consistent with the failure of CHO cells to exclude Trypan blue at this time point (26, 27). Tetanolysin itself did not introduce artifactual ADP-ribosylation, because it did not alter the pattern of labeled proteins seen after a 4-h infection. These experiments also established predominant labeling of 50- and 25-kDa proteins after a 3-h infection when cells were still viable.

Early experiments by Coburn et al. (10) revealed that in vitro ExoS modified a specific group of 20–30-kDa proteins that included Ras. Consistent with those findings, we observed after a 3-h infection with ExoS-producing P. aeruginosa, the specific ADP-ribosylation of a group of proteins between 20 and 30 kDa that included Ras. Comparison of immunoprecipitates from GFP-transfected CHO cells and HRas-transfected cells after the 3-h infection revealed that Ras was a minor component of radiolabeled endogenous proteins. We are attempting to identify other proteins modified at that time.

The 50-kDa band modified was identified as ExoS. Although auto-ADP-ribosylation of bacterial toxins has been noted in vitro, there are no reports that ADP-ribosylation alters toxin function. In mammalian cells, auto-ADP-ribosylation of ADP-ribosyltransferases ART5 (28) and NADase EC 3.2.2.5 (29) modulates NAD glycohydrolase activity. In addition, auto-ADP-ribosylation of an arginine-specific chicken heterophil ADP-ribosyltransferase was reported to alter intrinsic transferase activity (30). In amino acid sequence, ExoS more closely resembles eukaryotic ADP-ribosyltransferases than other bacterial ADP-ribosyltransferases (31). In vivo auto-ADP-ribosylation of ExoS and subsequent functional alteration establishes additional similarity with mammalian ADP-ribosyltransferases. Auto-ADP-ribosylation of ExoS modifies Arg-146, an arginine required for GAP activity. Modulation of Rho GAP activity by auto-ADP-ribosylation may reveal further differences between ExoS and ExoT, a type III-secreted P. aeruginosa effector which has GAP activity similar to ExoS, but lacks ADP-ribosyltransferase activity.

REFERENCES

1. Bodley, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) Rev. Infect. Dis. 5, 279–313
2. Frank, D. (1997) Mol. Microbiol. 26, 621–639
3. Gotor, U. M., Schmidt, G., Pederson, K. J., Aktories, K., and Barbieri, J. T. (1999) J. Biol. Chem. 274, 36369–36372
4. Ganesan, A. K., Vincent, T. S., Olson, J. C., and Barbieri, J. T. (1999) J. Biol. Chem. 274, 21823–21829
5. Pederson, K. J., Vailis, A. J., Aktories, K., Frank, D. W., and Barbieri, J. T. (1999) Mol. Microbiol. 32, 393–401
6. Pederson, K. J., Pai, S., Vailis, A. J., Frank, D. W., and Barbieri, J. T. (2000) Mol. Microbiol. 37, 298–299
7. Wurtele, M., Wolf, E., Pederson, K. J., Buchwald, G., Ahmadian, M. R., Barbieri, J. T., and Wittinghofer, A. (2001) Nat. Struct. Biol. 8, 23–26
8. Knob, D. A., Finck-Barbancon, V., Kulich, S. M., and Barbieri, J. T. (1995) Infect. Immun. 63, 3182–3186
9. Pederson, K. J., and Barbieri, J. T. (1998) Mol. Microbiol. 30, 751–759
10. Coburn, J., Wyatt, R. T., Iglewski, B. H., and Gill, D. M. (1988) J. Biol. Chem. 264, 9004–9008
11. Ganesan, A. K., Frank, D. W., Misra, R. P., Schmidt, G., and Barbieri, J. T. (1998) J. Biol. Chem. 273, 7332–7337
12. Moss, J., Ehrmantraut, M. E., Banwart, B. D., Frank, D. W., and Barbieri, J. T. (2001) Infect. Immun. 69, 1185–1188
13. Riese, M. J., Wittinghofer, A., and Barbieri, J. T. (2001) Biochemistry 40, 3289–3294
14. Ahnert-Hilger, G., Wegenhorst, U., Stecher, B., Spicher, K., Rosenthal, W., and Gratz, M. (1992) Biochem. J. 284, 321–326
15. Zhang, L., Wang, H., Masters, S. C., Wang, B., Barbieri, J. T., and Fu, H. (1999) Biochemistry 38, 12159–12164
16. Fu, H., Coburn, J., and Collier, R. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2320–2324
17. Aktories, K., Mohr, C., and Koch, G. (1992) Curr. Top. Microbiol. Immunol. 175, 115–131
18. Wurtele, M., Renault, L., Barbieri, J. T., Wittinghofer, A., and Wolf, E. (2001) FEBS Lett. 491, 26–29
19. Xu, Y., and Barbieri, J. T. (1995) Infect. Immun. 63, 825–832
20. McGuffie, E. M., Frank, D. W., Vincent, T. S., and Olson, J. C. (1998) Infect. Immun. 66, 2607–2613
21. Collmer, A. (1996) in ADP-ribosylating Toxins and G Proteins (Moss, J., and Vaughan, M., eds) American Society for Microbiology, Washington, D. C.
22. Sundin, C., Henriksen, M. L., Hallberg, B., Forberg, A., and Fritzis-Lindsten, E. (2001) Cell. Microbiol. 3, 237–246
23. Alava, M. A., DeBell, K. E., Conti, A., Hoffman, T., and Bonvini, E. (1992) Biochem. J. 284, 189–199
24. Conti, A., Brando, C., DeBell, K. E., Alava, M. A., Hoffman, T., and Bonvini, E. (1993) J. Biol. Chem. 268, 783–791
25. Aktories, K., Schmidt, G., and Just, I. (2000) Biol. Chem. 381, 421–426
26. Vallis, A. J., Finck-Barbancon, V., Yahr, T. L., and Frank, D. W. (1999) Infect. Immun. 67, 2040–2044
27. Olsen, J. C., McGuffie, E. M., and Frank, D. W. (1997) Infect. Immun. 65, 248–256
28. Stevens, L. A., Moss, J., Vaughan, M., Piazza, M., and Rappuoli, R. (1999) Infect. Immun. 67, 259–265
29. Han, M.-K., Lee, J.-Y., Cho, Y.-S., Song, Y. M., An, N.-H., Kim, H.-R., and Kim, J.-K. (2000) Curr. Top. Microbiol. Immunol. 237, 367–371
30. Knight, D. A., Finck-Barbancon, V., Kulich, S. M., and Barbieri, J. T. (1995) Infect. Immun. 63, 3182–3186
31. Ganesan, A. K., Mende-Mueller, L., Selzer, J., and Barbieri, J. T. (1999) J. Biol. Chem. 274, 9065–9070