Biochemical and Biophysical Research Communications **291,** 91–100 (2002)
doi:10.1006/bbrc.2002.6402, available online at http://www.idealibrary.com on IDEAL®

Eukaryotic Cell Determination of ExoS ADP-Ribosyltransferase Substrate Specificity

Jennifer E. Fraylick, Elizabeth A. Rucks, Deanne M. Greene, Timothy S. Vincent, and Joan C. Olson[1]

Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 165 Ashley Avenue, Suite 309, Charleston, South Carolina 29425

Received December 21, 2001

Exoenzyme S (ExoS) ADP-ribosylates multiple low-molecular-mass G- (LMMG-) proteins *in vitro*. Identification of the *in vivo* substrate specificity of ExoS has been hindered by its bacterial contact delivery into eukaryotic cells and difficulties in identifying ADP-ribosylated proteins within cells. Two-dimensional electrophoresis comparisons of substrate modifications by ExoS *in vitro* to that following bacterial translocation into HT-29 epithelial cells identified Ras, Ral, and Rab proteins and Rac1 as *in vivo* substrates of ExoS ADPRT activity. Cellular fractionation studies identified a relationship between membrane association and efficiency of substrate modification. Moreover, Rac and Cdc42 relocalized to the membrane in response to ExoS. Comparisons of substrate modification to time of exposure to ExoS identified a progression of substrate modification, with Ras, RalA, and Rab5 modified first, followed by Rab8 and 11, then Rab7 and Rac1. The data support that intrinsic properties of LMMG-proteins and their subcellular localization are determinants of bacterially translocated ExoS substrate selectivity.

Key Words: **exoenzyme S; ADP-ribosylation; type III secretion; *Pseudomonas aeruginosa;* LMMG-proteins; HT-29 epithelial cells; two-dimensional electrophoresis.**

Exoenzyme S (ExoS) is directly translocated into eukaryotic cells by the opportunistic pathogen, *Pseudomonas aeruginosa*, through the contact-dependent type III secretory process (1). Bacterial-eukaryotic cell co-culture systems that compare the effects of ExoS producing and non-ExoS producing isogenic strains have identified effects of bacterially translocated ExoS on epithelial cell DNA synthesis, morphology, microvilli effacement, adhesion, and microbial invasion (2–4). ExoS has also been associated with anti-phagocytic effects on macrophage (5) and immune stimulatory effects on lymphocytes (6). The diverse cellular effects of ExoS are believed to relate to its bifunctional mode of action. ExoS includes a GTPase activating (GAP) activity in its amino-terminus that, *in vitro,* targets low-molecular-mass G- (LMMG-) proteins in the Rho family (7). The carboxy-terminus of ExoS includes an ADP-ribosyltransferase (ADPRT) activity (8) that, *in vitro,* preferentially targets LMMG-proteins in the Ras and Rab families, including Ras, Rap, Ral, and Rab3, 4, 5, 6, and 7 (9, 10). When GAP or ADPRT mutant forms of ExoS were used to examine the relative roles of its two functional domains in the cellular effects of bacterially translocated ExoS, its ADPRT activity was found to be required for alterations in epithelial cell growth and morphology, while the direct role of the GAP activity was less obvious (11). These results focused attention on substrates targeted by ExoS ADPRT activity *in vivo* in determining its cellular mechanism of action.

It is inherently difficult to identify substrates of ExoS ADPRT activity following bacterial translocation due to experimental limitations in identifying ADP-ribosylated proteins within cells. The recognition of a shift in protein mass following the co-culture of eukaryotic cells with ExoS producing bacteria provided an initial indication that Ras was an *in vivo* target of ExoS ADPRT activity. The ADP-ribosylation of cellular Ras by bacterially translocated ExoS was further supported by two-dimensional electrophoresis (2DE), which identified alterations in isoelectric point (p*I*) and mass consistent with the transfer of an ADP-ribose moiety, and by intracellular NAD labeling experiments (12, 13). The ADP-ribosylation of Ras by ExoS was subsequently found to interfere with the Ras-Raf-1 signaling pathway, identifying a possible cellular mechanism for the effects of ExoS on DNA synthesis and cell morphology (13–15). In similar analyses of the ADP-ribosylation of Rap1 by bacterially translocated

[1] To whom correspondence and reprint requests should be addressed at Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 165 Ashley Avenue, Suite 309, P.O. Box 250908, Charleston, SC 29425. Fax: 843-792-0368. E-mail: olsonj@musc.edu.

0006-291X/02 $35.00

ExoS, no modification was detected (12). However, when Rap1 was expressed by transient transfection, modification was detected (16), confirming the potential for Rap1 to be ADP-ribosylated by ExoS within the cell. More recently, RalA was confirmed as an *in vivo* substrate of bacterially translocated ExoS ADPRT activity, and the cellular activity of RalA was found to be affected by its ADP-ribosylation by ExoS (17, 18).

To extend an understanding of the cellular mechanism of action of ExoS we have further explored the *in vivo* substrate specificity of ExoS ADPRT activity and examined cellular factors that might contribute to this specificity. Our studies support that the targeting of ExoS ADPRT activity for endogenous LMMG-proteins substrates is influenced by their subcellular localization and by the trafficking pattern of ExoS as it is translocated by the type III secretory process through the membrane into the cytosol.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

P. aeruginosa strains used in these studies were kindly provided by Dara Frank (Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI) and include the parental strain 388 (19) and strain 388Δ*exoS* (388ΔS), an isogenic mutant that lacks production of ExoS (20). Strain 388ΔS complementation studies (3) and studies of the non-ExoS producing *P. aeruginosa* strain, PA103 (11), have been used to confirm ExoS specific effects on cell function. Bacterial strains were grown for 16 h in ExoS induction medium (TSBD-N) (19) in preparation for co-culture with eukaryotic cells, as previously described (2). Bacteria were then diluted, based on culture OD_{590}, to approximately 10^7 CFU/ml in tissue culture medium containing 0.6% bovine serum albumin (Sigma, St. Louis, MO) and added to eukaryotic cells at a multiplicity of infection (m.o.i.) of 50.

Eukaryotic Cell Culture

HT-29 colon carcinoma cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were maintained as specified by ATCC at 37°C in 5% CO_2–95% air in McCoy's 5A medium containing 10% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD) (McCoy's–FBS). In preparation for culture with bacteria, HT-29 cells were detached with 0.05% trypsin–0.53 mM EDTA (Gibco-BRL) (trypsin–EDTA), seeded at 1×10^5 cells/ml and cultured for 48 h, reaching 50–60% confluency. Cells were then cultured with no bacteria or strain 388 or 388ΔS in McCoy's–0.6% BSA for 2 to 6 h, as indicated, and assayed for modification of eukaryotic LMMG-proteins. HT-29 cells were selected for these studies because they maintain many characteristics of normal epithelial cells, including the ability to form a polarized monolayer (21), supporting their use as model to understand the *in vivo* substrate specificity of ExoS during the *P. aeruginosa* infectious process.

LMMG-Protein Expression and Purification

H-Ras was produced and purified as previously described (13). Rac1 cDNA, obtained from the Guthrie cDNA Resource Center (Guthrie Research Institute, Sayre, PA), was amplified using the primers, forward 5′-GGAATTCCATATGCAGGCCATCAAGTGTG-TGG-3′, and reverse 5′-CGCGGATCCTTACAACAGCAGGCATTTT-CTC-3′ and was inserted into pET15b using *Nde*I and *Bam*HI sites included in the primers. This allowed the expression and purification of Rac1 as a His-tagged protein, using previously described methods (22).

Analysis of in Vivo Targets of ExoS ADPRT Activity

SDS–PAGE analysis of LMMG-protein modification. ADP-ribosylation of endogenous LMMG-proteins was initially assessed based on an alteration in protein mobility by SDS–PAGE following exposure of HT-29 cells to strain 388 or 388ΔS. For analysis of Ras modification, Ras was immunoprecipitated from cell extracts using monoclonal Y13-259 Ras antibody as previously described (12), resolved by SDS 12%–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Ras was detected with mouse anti-H-Ras LA069 or anti-Pan-Ras LA055 (Quality Biotech, Camden, NJ) and visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL) (ECL). For analyses of modification of RalA, Rab4, Rab5, Rab7, Rab8, Rab11, Rac1, Cdc42, and RhoA, B, C, cells were lysed in Laemmli electrophoresis buffer (23) and heated at 95°C for 5 min. Proteins were resolved by SDS 12%–PAGE, and detected using mouse monoclonal antibodies against Rac1, Rab4, Rab8, and Rab11 (Transduction Laboratories, Lexington, KY), or rabbit polyclonal antibodies against Rab5b, Rab7, Cdc42, and RhoA, B, C (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by ECL.

Two-dimensional analysis of LMMG-protein modification. For 2DE analysis, HT-29 cells were lysed following exposure to bacteria in 2DE lysis buffer [8 M urea, 2% Triton X-100, 1% DTT, 1.5% Pharmalytes (Pharmacia, Piscataway, NJ)] for 30 min on ice. Samples in 2DE lysis buffer were then used to rehydrate immobilized pH gradient gel strips (7 cm, pH 3–10; Pharmacia) overnight, or gel strips were rehydrated in 2DE lysis buffer and samples were cup-loaded onto strips. Proteins were focused based on p*I* for 16 to 24 h at 3500 V (Ras and Rab) or 2000 V (Rac). Strips were then equilibrated in SDS equilibration buffer (50 mM Tris, pH 6.8, 6 M urea, 30% glycerol, 2% SDS) containing 20 mg/ml DTT for 20 min, followed by equilibration in SDS equilibration buffer containing 25 mg/ml iodoacetamide for 20 min. Proteins were resolved in the second dimension by SDS 12%–PAGE, transferred to PVDF membranes, and immunoblotted for Rab5, Rab11, Rac1, Cdc42, or Rho A, B, C, as described above. An internal control (LA059 or LA060 monoclonal antibody, Quality Biotech) was included with each sample to allow alignment of specific LMMG-proteins.

Analysis of ADP-Ribosylation of LMMG-Proteins by ExoS in Vitro

In vitro ExoS ADPRT reactions were performed in 0.2 M Tris acetate, pH 6.0, containing, 0.2 μM purified His-tagged ExoS (12), 10 mM NAD, 1 mM $MgCl_2$, a source of 14-3-3 (either 0.2 μM 14-3-3ξ (Upstate Biotechnology, Lake Placid, NY), 10 μl of wheat germ extract, or endogenous 14-3-3 in cell extracts) in a 100-μl volume, and reactions were incubated for 30 or 60 min at 25°C. *In vitro* ADPRT reactions of Rac1 or H-Ras included 1 μg of purified protein loaded with 10 μM GTPγS, according to the procedure of Antonny *et al.* (24). *In vitro* ADPRT reactions of Rab proteins used 50 μl of HT-29 cell lysate as the source of cellular Rab and included protease inhibitors [1 mM PMSF, 10 μg/ml, each, leupeptin and aprotinin (Sigma)]. For SDS–PAGE analyses, reactions were stopped with 4× Laemmli sample buffer and heating at 95°C for 2 min. For 2DE analyses, reactions were equilibrated in 2DE lysis buffer. Samples were resolved and analyzed for specific LMMG-proteins as described above.

Analysis of ExoS ADPRT Substrate Modification in Fractionated Cells

Following a 5-h exposure to strains 388 or 388ΔS, HT-29 cell monolayers were washed with PBS, detached with trypsin–EDTA, resuspended in McCoy's–FBS to inactivate the trypsin, washed once with PBS, then once with homogenization medium (20 mM Tris–Cl,

pH 7.4, 250 mM sucrose, 1 mM EDTA, and protease inhibitors). Each wash was followed by centrifugation at 400*g* for 5 min. Cell pellets were resuspended in homogenization medium and homogenized on ice with 16 strokes of a Potter–Elvehjem homogenizer. The lysate was centrifuged at 1000*g* for 10 min to remove unfractionated cells and nuclei, and the supernatant was removed and centrifuged at 116,000*g* for 1 h. This resulted in a S100 supernatant (cytosol) and P100 pellet (membrane) fraction which were assayed for substrate modification as described above.

Time-Course Analysis of ExoS Substrate Modification

Comparisons of LMMG-protein substrate ADP-ribosylation, relative to time of exposure to bacteria, were performed in parallel on samples obtained following a 2-, 3-, 4-, or 5-h coculture period. Specific LMMG-proteins were assayed for substrate modification by SDS–PAGE, as described above. To quantify the relative efficiency of LMMG-protein modification by ExoS, densitometry values of immunoblot images were obtained using the NIH image version 1.6 program. Percentage-modified protein was calculated relative to total modified plus unmodified protein.

RESULTS

Modification of Endogenous LMMG-Proteins by Bacterial Translocation of ExoS

To better understand the mechanism of ExoS effects on eukaryotic cell function, endogenous LMMG-proteins were screened for possible ADP-ribosylation by ExoS based on altered mobility by SDS–PAGE analysis, following exposure of HT-29 cells to ExoS producing bacteria. Differences were detected in the shift in molecular mass and the efficiency of modification of the individual LMMG-proteins (Fig. 1A). Ras, a previously identified *in vivo* substrate of ExoS ADPRT activity (12) was included in these studies for comparison. In the Rab family, Rab5 showed two shifts in molecular mass, while a single shift in mass was detected for Rab7, Rab8 and Rab11, and no shift in mass was detected for Rab4. The two modified forms of Rab5 detected by SDS–PAGE were consistent with two sites of ADP-ribosylation by ExoS. The single shift in mass detected for Rab7, Rab8, and Rab11 may relate to one or more sites of ADP-ribosylation, for Ras, which exhibits a single shift in mass, is known to be ADP-ribosylated by ExoS at two or three sites (13, 25). The lack of shift in mass of Rab4 suggests that it differs from the other Rab proteins in its ability to be ADP-ribosylated by bacterially translocated ExoS.

Analyses of the Rho family of LMMG-proteins identified a single shift in the mass of Rac1 following bacterial translocation of ExoS, but no alteration in the mobility of Cdc42 or RhoA, B, C was detected (the Rho proteins resolving as three distinct bands). The results suggest that Rac1, but not Cdc42 or RhoA, B, C, can be ADP-ribosylated by bacterially translocated ExoS.

Together, SDS–PAGE analyses draw attention to differences in the modification of LMMG-proteins in the Ras, Rab, and Rho families by bacterially translocated ExoS. Some proteins do not appear to be modified

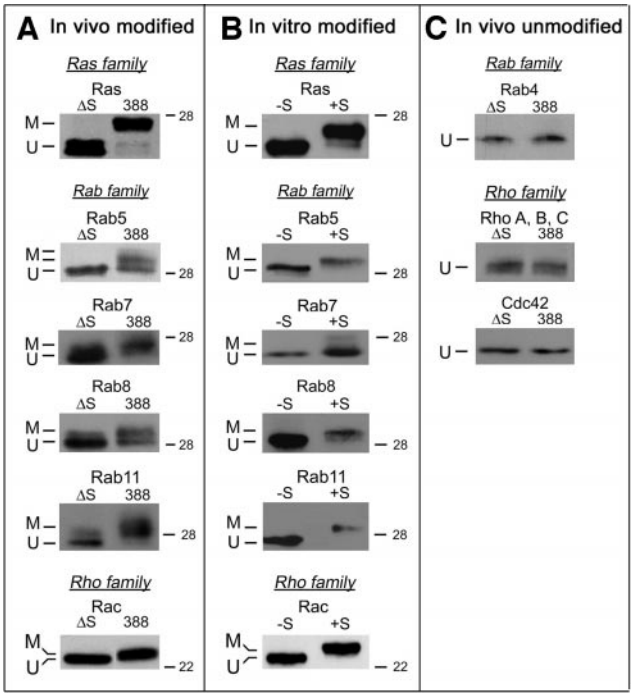

FIG. 1. SDS–PAGE analysis of ADP-ribosylation of LMMG-proteins *in vivo* and *in vitro*. (A, C) *In vivo analysis:* HT-29 cells were grown to 50–65% confluency and cocultured with 1×10^7 CFU/ml 388 or 388ΔS (ΔS) for 4–5 h. To examine Rab5, Rab7, Rab8, Rab11, Rac1, Cdc42, and Rho A, B, C modification, cells were lysed in Laemmli sample buffer and resolved by SDS 12%–PAGE, blotted onto PVDF membranes and probed with an antibody to the indicated protein, as described under Materials and Methods. To examine Ras modification, cells were lysed in TBS-TDS, immunoprecipitated with Y13-259 (anti-Pan Ras) antibody, then resolved on SDS–PAGE and probed with H-Ras antibody. Blots were developed using enhanced chemiluminescence (ECL). (B) *In vitro analysis:* To compare ADP-ribosylation of specific proteins by ExoS *in vivo* to that detected *in vitro*, 1 μg of purified protein (Rac1 or H-Ras) or 50 μl of HT-29 cell extract (Rab proteins) were incubated for 30 or 60 min at 25°C in 0.2 M Tris–acetate, pH 6.0, containing 0.2 μM purified ExoS, 1 mM $MgCl_2$, 10 mM NAD, and a source of 14-3-3. Reactions were stopped with 4× Laemmli sample buffer and heating at 95°C, resolved and analyzed for specific proteins as in A. The mobilities of modified (M) and unmodified (U) proteins are indicated, and a molecular mass reference is included. The results are representative of analyses performed in multiple independent studies.

by ExoS *in vivo*, which may, as in the case of RhoC (10), relate to intrinsic protein properties that preclude their functioning as substrates of ExoS. Other proteins exhibit distinctly different shifts in mass upon exposure to ExoS, which could relate to the number or specific sites of ADP-ribosylation by ExoS in the individual proteins. Alternatively, it also remains possible that ExoS might induce other cellular post-translational modifications that affect protein mobility.

Comparison of in Vivo and in Vitro Modification of LMMG-Proteins by ExoS

One approach that lends further support to the possibility that alterations in LMMG-protein mobility de-

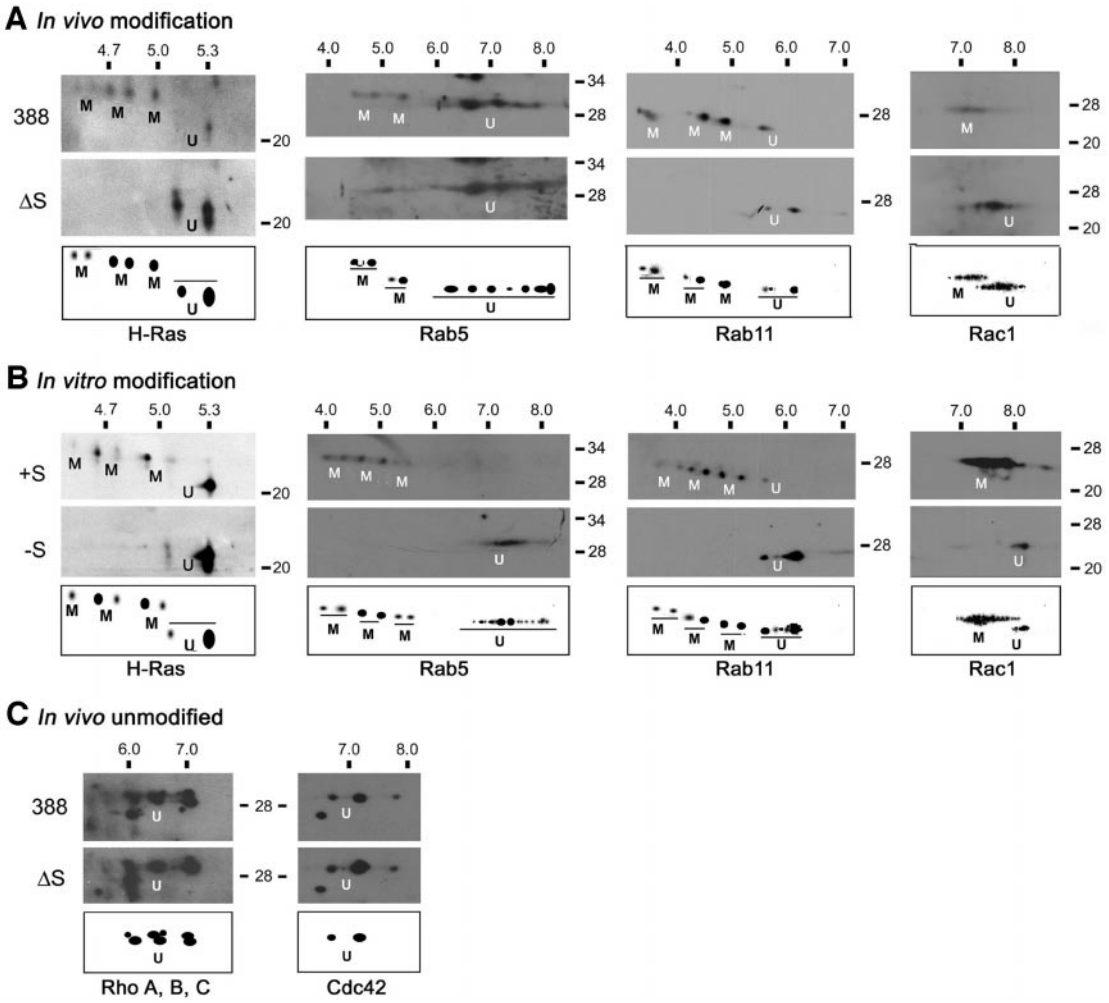

FIG. 2. 2DE analysis of LMMG-protein modification by ExoS *in vivo* and *in vitro.* (A, C) *In vivo:* Following a 5-h coculture period with strain 388ΔS (ΔS) or 388, HT-29 cells were lysed in 2DE lysis buffer and lysates were focused on 7-cm, pH 3–10 Immobiline dry strips equilibrated in SDS equilibration buffer then separated in the second dimension by SDS 12%–PAGE. Following transfer to PVDF membranes, individual LMMG-proteins were probed with protein specific antibodies and visualized as in Fig. 1. 2DE analyses of non-bacterial treated cell lysates were identical to that of 388ΔS treated bacteria (not shown). (B) *In vitro:* ADP-ribosylation of the indicated proteins by purified ExoS was performed as described in the legend to Fig. 1B. Reactions were equilibrated in 2DE lysis buffer and resolved by 2DE as described above. Interpretations of the modifications of each LMMG-protein are diagrammatically represented below the respective blots. Unmodified (U) and modified (M) proteins are labeled. The mass is indicated at the sides of the panels and p*I* above. Data are representative of two or more independent analyses.

tected following exposure to ExoS producing bacteria relate to the transfer of an ADP-ribose moiety, is the detection of similar alterations in protein mobility following *in vitro* ExoS ADPRT reactions. As shown in Fig. 1B, similar qualitative shifts in the mass of Ras, Rab, and Rac1 proteins were detected when the individual proteins were ADP-ribosylated by purified ExoS *in vitro* and compared to that following bacterial translocation of ExoS. However, differences were observed in the efficiency of modification, with Rab7 being more efficiently modified *in vivo,* while Rab5, Rab8, Rab11 and Rac1 appeared more efficiently modified *in vitro.* The detection of Rab and Rac modification following ADP-ribosylation by ExoS *in vivo* and *in vitro* further supports that these proteins are substrates of ExoS ADPRT activity *in vivo.* However, an inability to determine the molecular basis for differences observed in the mobility in these proteins, based on SDS–PAGE analysis, precludes confirmation of this reactivity.

Two-Dimensional Electrophoresis Analysis of the ADP-Ribosylation of LMMG-Proteins by ExoS

2DE resolves proteins based on mass and p*I.* This technique was previously used to confirm the *in vivo* ADP-ribosylation of endogenous H-Ras at three sites by bacterially translocated ExoS (13) (refer to Fig. 2), which coincided with three sites of H-Ras ADP-ribosylation by ExoS *in vitro,* at Arg41 (a preferred site), Arg128 (a secondary site) and Arg135 (an alternative

site) (25, 26). To further support that the altered mobility of Rab and Rac1 proteins by bacterially translocated ExoS related to the transfer of an ADP-ribose moiety, the modification of Rab5, Rab11, and Rac1 by ExoS *in vivo* and *in vitro* was compared by 2DE. In addition, RhoA, B, C, and Cdc42 were examined by 2DE for modification by bacterially translocated ExoS to assess whether the modification of any of these proteins could be detected by this method of analysis.

As represented for H-Ras in Fig. 2, LMMG-proteins often resolve as several spots by 2DE, which reflect their different post-translational forms (27). In the analysis of cellular Ras modification by bacterially translocated ExoS, Ras isoforms were found to shift in groups, and three shifts in isoform groups were detected for H-Ras (the third, alternative site of modification being weak), indicating three sites of ADP-ribosylation (13). 2DE analyses of cellular Rab5, Rab11, and Rac1 also found these LMMG-proteins to focus as several spots or protein bands. Following exposure to ExoS producing bacteria, Rab5 isoforms exhibited an approximate 2 kDa shift in mass, which was accompanied by 2 shifts in p*I* of the isoform group of approximately 0.5 p*I* units each (Fig. 2A). The shifts in mass and p*I* of Rab5 observed *in vivo* corresponded closely with that observed following the ADP-ribosylation of cellular Rab5 by purified ExoS *in vitro,* with the exception that a third modified form of Rab5 was detected *in vitro* (Fig. 2B). The results support that Rab5 is ADP-ribosylated at two sites by bacterially translocated ExoS. When Rab11 modification by ExoS was compared by 2DE, again similar shifts in mass and p*I* were detected following exposure to ExoS *in vivo* and *in vitro,* supporting that Rab11 was also a substrate of bacterially translocated ExoS ADPRT activity. Three shifts in p*I* of Rab11 of about 0.3 to 0.6 p*I* units were detected, consistent with three sites of ADP-ribosylation of Rab11 by ExoS *in vivo.* Rac1 was more difficult to focus by 2DE, focusing as a band, rather than distinct isoform spots. Following bacterial translocation of ExoS, Rac1 exhibited a single shift in mass (~550 Da), accompanied by a single shift in p*I* of 0.6 to 0.8 p*I* units. A similar shift in mass and p*I* was detected following the ADP-ribosylation of purified Rac1 by purified ExoS *in vitro.* The interpretation of these results is that Rac1 is ADP-ribosylated by bacterially translocated ExoS at one site.

2DE analyses of cellular RhoA, B, C revealed multiple spots and varying molecular masses corresponding with the three Rho proteins. Multiple cellular isoforms of Cdc42 were also detected by 2DE. No shifts in mass or p*I* were evident in Rho or Cdc42 following exposure to ExoS producing bacteria, further supporting that these proteins were not preferred substrates of ExoS ADPRT activity upon its bacterial translocation.

Cellular Localization of in Vivo Substrates of ExoS ADPRT Activity

Differences detected in the ADP-ribosylation of proteins in the Ras, Rab and Rho families by bacterially translocated ExoS drew attention to the possibility that factors other than intrinsic protein properties might be contributing to substrate modification by ExoS within the cell. Of specific note in this regard was the lack of ADP-ribosylation of Rab4 *in vivo,* which was previously reported to be ADP-ribosylated by ExoS *in vitro* (10) and in SLO permeabilized reticulocytes (28). We similarly detected a shift in the mass of Rab4 by ExoS based on immunoblot analyses following an *in vitro* ADPRT reaction (data not shown). The finding that Rab4 can function as a substrate of ExoS *in vitro,* yet is not ADP-ribosylated by ExoS *in vivo,* indicates that cell associated factors influence the availability of endogenous Rab4 to ADP-ribosylation by bacterially translocated ExoS. Also, notable was the apparent selective ADP-ribosylation of endogenous Rac1 by bacterially translocated ExoS, but not other Rho family proteins, RhoA, B, C and Cdc42. In our previous studies we found Ras, which is plasma membrane associated (29) to be more efficiently modified by ExoS *in vivo* than Rap1a (12), which is localized to the endocytic and lysosomal vesicles (30). Since both Ras and Rap1 are substrates of ExoS ADPRT activity *in vitro,* this led to the notion that cellular localization might contribute to the targeting specificity of bacterially translocated ExoS.

To examine the relationship between the efficiency of LMMG-protein ADP-ribosylation by bacterially translocated ExoS and cellular localization, HT-29 cells were fractionated into an S100, cytosol and P100, membrane fraction following coculture with ExoS producing bacteria. As shown in Fig. 3, LMMG-proteins were distributed differently between the cytosolic and membrane fractions, with the most efficient substrate modification consistently detected in the membrane fraction. In the Ras family, RalA, previously identified as an *in vivo* substrate of ExoS ADPRT activity (17), preferentially localized to the membrane fraction, where it was efficiently modified. In the Rab family, Rab5, like Rab7 and Rab8 (not shown), were somewhat equally distributed between the cytosolic and membrane fraction, but the most efficient modification by ExoS was detected in the membrane fraction. Rab4, primarily a cytosolic protein appeared to further localize to the cytosol in response to ExoS producing bacteria, and was not modified by ExoS. In the Rho family, Rac and Cdc42, which normally localize to the cytosol, were found to re-localize to the membrane fraction when exposed to ExoS producing bacteria, where modification was detected. Re-localization of RhoA, B, C to the membrane was not evident in the same fractions, and Rho remained unmodified. The detection of modi-

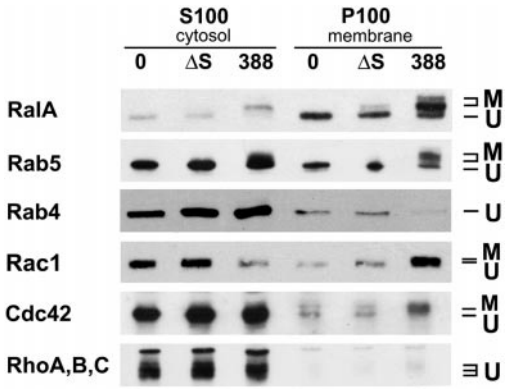

FIG. 3. HT-29 cell fractionation and analysis of the ADP-ribosylation of LMMG-proteins. HT-29 cells were grown to ~60% confluency in T75 culture flasks and co-cultured with 10^7 CFU/ml 388 or 388ΔS (ΔS) for 5 h. Following the coculture period, HT-29 cells were washed, trypsinized, resuspended in homogenization buffer and disrupted as described under Materials and Methods. Cell mixtures were centrifuged at 116,000*g* for 1 h, resulting in a cytosolic (S100) and membrane (P100) fraction. Samples of each fraction were resolved by SDS 12%–PAGE and the indicated proteins detected as in Fig. 1. The results compare substrate modification from a single experiment and are representative of results obtained from two or more independent studies. The mobilities of modified (M) and unmodified (U) proteins are indicated.

fication of Cdc42 in the P100 membrane was unexpected, considering our previous inability to detect modification of endogenous Cdc42. In direct comparisons of Cdc42 and Rac1 in the P100 fraction, a much smaller portion of Cdc42 was found to be localized to the membrane and modified. We interpret the inability to consistently detect Cdc42 modification *in vivo* to relate to its less efficient modification and difficulties in recognizing its modification in the presence of a much larger portion of unmodified, cytosolic Cdc42.

Time-Course Evaluation of Substrate Modification by ExoS ADPRT Activity in Vivo

Fractionation studies supported that the efficiency of LMMG-protein ADP-ribosylation by ExoS related to the efficiency in which the respective substrates localized to the membrane. The more efficient modification of membrane associated proteins would be consistent with the type III mediated translocation of ExoS through the membrane. To gain further insight into the trafficking pattern of bacterially translocated ExoS relative to substrate modification, the efficiency of specific LMMG-protein ADP-ribosylation was monitored with increasing time of exposure to ExoS producing bacteria. As shown in Fig. 4A, Ras and RalA, primarily plasma membrane proteins, were the first to exhibit shifts in mass, which occurred after a 3-h exposure to bacteria. The ADP-ribosylation of Rab proteins corresponded closely with the localization of their respective endosomal compartments within the cell relative to the plasma membrane. A faint shift in Rab5, which is associated with early endosomes, was detected at 3 h. Shifts in Rab8 and Rab11, which are associated with endocytic recycling, were apparent at 4 h. Efficient modification of Rab7, which is associated with late endosomes, was not detected until 5 h. Rac1 modification was most evident after a longer, 5-h exposure to bacteria, indicating that its cycling to the membrane in association with ADP-ribosylation by ExoS required a longer exposure to bacteria. The efficiency of substrate modification was quantified and is graphically repre-

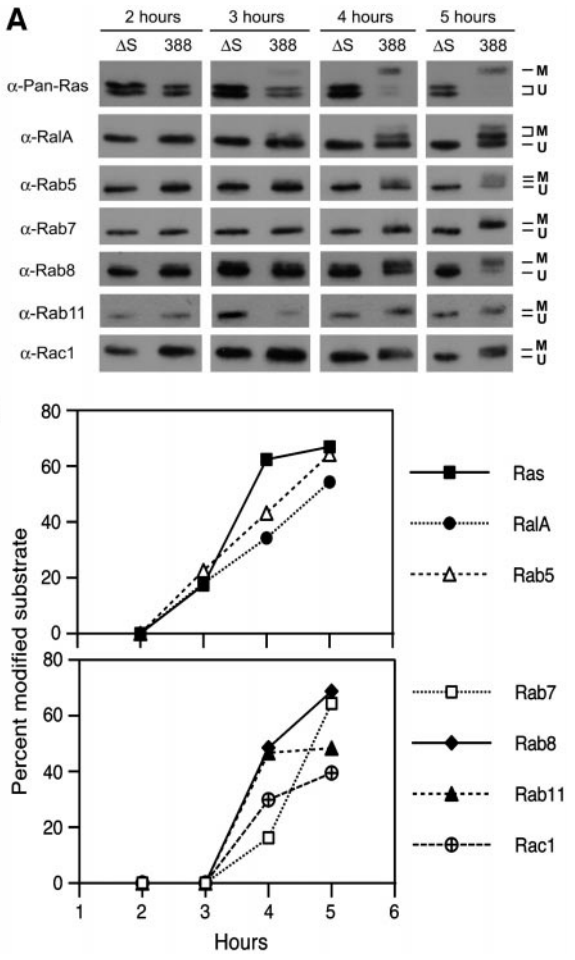

FIG. 4. LMMG-protein modification by ExoS relative to time of exposure to bacteria. HT-29 cells were cocultured with strain 388 or 388ΔS (ΔS) for 2–5 h. (A) To examine Ras modification, cells were lysed in 1 ml TBS-TDS, and Ras was immunoprecipitated and detected as in Fig. 1. To examine the modification of RalA, Rab5, Rab7, Rab8, Rab11, and Rac1, cells were lysed in 1× Laemmli sample buffer, and the indicated proteins were resolved and detected as in Fig. 1. The results compare substrate modification on the same samples from one experiment, and are representative studies repeated three times. Mobilities of modified (M) and unmodified (U) proteins are indicated. (B) The relative efficiency of LMMG-protein modification by ExoS was quantified based on densitometry values of immunoblot images obtained using the NIH image version 1.6 program. Percentage-modified protein was calculated relative to total modified plus unmodified protein.

sented relative to the time of exposure to bacteria in Fig. 4B. The comparison shows the hierarchical order and rate of modification of specific LMMG-proteins, with all substrates showing a high degree of modification after 5 h.

DISCUSSION

The requirement for ExoS ADPRT activity for effects of bacterially translocated ExoS on epithelial cell growth and morphology directed attention to the importance of determining the *in vivo* substrate specificity of ExoS ADPRT activity in understanding the cellular mechanism of action of ExoS. Confirmation of cellular proteins ADP-ribosylated by bacterially translocated ExoS has proven to be technically difficult. Unlike analyses of phosphorylated proteins, no antibodies are currently available to detect ADP-ribosylated proteins. In addition, NAD, the source of the ADP-ribose in the ExoS ADP-ribosylation reaction, is not internalized by eukaryotic cells, precluding the direct detection of ADP-ribosylated proteins within cells using a radiolabeled NAD probe. Alternative approaches of introducing radiolabeled NAD into cells include altering cell membrane permeability to allow internalization of NAD, or radiolabeling intracellular NAD pools using radiolabeled adenine or adenosine. The problem with approaches that alter cell membrane permeability is that these alterations would also affect the internalization of ExoS, thus questioning whether substrate modification resulted from bacterially translocated or diffused ExoS. Analyses using adenine or adenosine to radiolabel intracellular NAD pools are also problematic because of their association with high background labeling and, in turn, inefficient labeling of specific immunoprecipitated proteins (as evident by long, ~1 month, exposure times to detect bands in autoradiographs) (12). Based on these experimental difficulties, we chose to use 2DE to assess substrate modification by bacterially translocated ExoS. This method offers the advantage of not requiring alterations in membrane permeability to detect cellular substrate modification. Evidence that protein modifications detected by this method result from the transfer of an ADP-ribose moiety is derived from the identification of similar alterations in both p*I* and mass in comparisons of *in vivo* and *in vitro* ExoS ADPRT reactions. The finding that individual proteins exhibited distinctly different p*I* and mass modification patterns following their ADP-ribosylation by ExoS provided further corroboration that the detected modifications did indeed result from the transfer ADP-ribose moieties, rather than other cellular posttranslational modifications.

In the process of identifying LMMG-proteins targeted by bacterially translocated ExoS, it became apparent that cellular factors were influencing the order and efficiency of substrate modification by ExoS. Within the Ras and Rab families, studies described here combined with previous studies (12, 17), identified endogenous Ras, RalA, Rab5, Rab11, and likely Rab7 and Rab8, but not Rab4 nor Rap1, as *in vivo* substrates of bacterially translocated ExoS. In the Rho family, endogenous Rac1 was identified as an *in vivo* substrate of ExoS, with Cdc42 modification being less efficient, and no modification of RhoA, B, C was detected. In all instances, membrane localization of the endogenous proteins was associated with more efficient ADP-ribosylation by ExoS. The preference for membrane-associated substrates is consistent with the type III mediated translocation of ExoS through the eukaryotic cell membrane and the increased accessibility of substrates in the plasma membrane to ExoS. Previous studies, however, also found the membrane microenvironment to enhance the enzymatic efficiency of ExoS ADPRT activity relative to substrate modification (11). It therefore remains possible that both increased accessibility and factors linked to membrane association might contribute to the more efficient substrate modification detected in the membrane fraction.

The association between membrane localization and the efficiency of ADP-ribosylation of LMMG-proteins by ExoS provided insight into why certain LMMG-proteins, such as Rab4, were not ADP-ribosylated by ExoS *in vivo*. Rab4 was previously identified as an *in vitro* substrate of ExoS ADPRT activity based on immunoblot analyses (10) (Rucks, unpublished data) and was found to colocalize with 14-3-3ζ in endocytic vesicles (28). Therefore the lack of detectable ADP-ribosylation of Rab4 by bacterially translocated ExoS could not be explained by intrinsic protein properties, or the sequestration of Rab4 from ExoS cofactor within the cell. Alternatively, cell fractionation studies found that Rab4 was only weakly membrane associated, and actually appeared to further localize to the cytosolic fraction upon exposure to ExoS producing bacteria, which corresponded with its lack of ADP-ribosylation. A relationship between substrate localization and modification in response to ExoS producing bacteria was also evident in analyses of the ADP-ribosylation of LMMG-proteins in the Rho family. While Rho family proteins, RhoA, B, C, Rac1 and Cdc42 reside mostly within the cytosolic fraction under normal cellular conditions, Rac1 and Cdc42 appeared to re-localize to the membrane fraction upon exposure to ExoS producing bacteria, where they were modified (albeit Cdc42 was modified with low efficiency). Conversely, RhoA, B, C, which did not relocalize to the membrane, were not ADP-ribosylated by ExoS. With regard to these Rho proteins, however, it remains possible that intrinsic properties might also contribute to their inefficient ADP-ribosylation by ExoS, as documented for RhoC (10).

The cytosolic and membrane association of Rab and Rho family proteins has been found to be linked to their

cycling between inactive GDP-bound and active GTP-bound forms (29). Rab and Rho proteins are maintained in the cytosol in the GDP-bound form through their association with GDI regulator proteins, which inhibit the dissociation of GDP. The release of GDI from the GDP-bound protein is coupled with translocation of the LMMG-protein to the membrane and its conversion to the GTP-bound form. Consistent with the possibility that GTP-binding by Rab proteins might contribute to their more efficient ADP-ribosylation by ExoS in the membrane fraction, Rab5 was found to be more efficiently ADP-ribosylated *in vitro* in its GTP compared to GDP-bound form (31). This same mechanism is not likely to apply to the enhanced modification of Ras and Ral by ExoS in the membrane fraction, since no preferential ADP-ribosylation of GTP- or GDP-bound Ras or Ral has been detected *in vitro* (13, 26) (Greene, unpublished observation). In addition, unlike Rho and Rab proteins, neither Ras nor Ral cycle between the cytosol and membrane in association with their GDP- or GTP-bound state (29).

Within the Rho family, the confirmation of Rac1 and implication of Cdc42 as *in vivo* substrates of ExoS ADPRT activity adds an interesting complexity to the cellular mechanism of ExoS, since both proteins are also known to be targeted by ExoS GAP activity *in vitro* (7). The sequestering of both Rac and Cdc42 at the membrane following exposure to ExoS producing bacteria indicates that ExoS has an effect on the function of these LMMG-proteins. The effect of ExoS on Rac and Cdc42 recycling to the cytoplasm may relate to ExoS associated interference of GTP hydrolysis or the interaction of their GDP-bound form with RhoGDI. While the mechanism for the effects of ExoS ADPRT activity on Rac or Cdc42 function have not been experimentally determined, the implications of these findings are that ExoS GAP and ADPRT activity may have coordinated effects on the function of Rac and Cdc42.

In comparing the ADP-ribosylation of endogenous LMMG-proteins by ExoS, it became apparent that not only the number of sites of ADP-ribosylation varied among the individual LMMG-proteins, but that distinct differences were observed in how specific proteins interpreted the addition of an ADP-ribose moiety. These differences were apparent in shifts in mass that range from 500 to 2000 daltons, and shifts in p*I* that range from 0.25 to 0.8 p*I* units per ADP-ribose addition. Also evident in 2DE analyses were differences in the number of sites of ADP-ribosylation by ExoS in individual LMMG-proteins. Studies described here support that bacterially translocated ExoS can ADP-ribosylate Rab11 at three sites, Rab5 at two sites, and Rac1 at one site. While the functional significance of these differences is unclear, it is known that the ADP-ribosylation of LMMG-proteins by ExoS can affect their function. Relative to Ras, the most extensively characterized *in vivo* substrate of ExoS ADPRT activity, ADP-ribosylation at Arg41 is known to interfere with GEF catalyzed guanine nucleotide exchange (14) and associated with the interruption of Ras-Raf1 signal transduction pathways (13–15). The cellular consequences of ADP-ribosylation of H-Ras at secondary sites, Arg128 or Arg135, remain unknown. The ADP-ribosylation RalA at specific residues by bacterially translocated ExoS has also been shown to interfere with its activation and downstream signaling (18), confirming an effect of ExoS on Ral function. Studies in *Xenopus* oocytes found that the ADP-ribosylation of Rab5 by ExoS interfered with endosome-endosome fusion, and related this to interference of Rab5 interaction with its downstream effector EEA1 using rat endocytic vesicles (31). Rab5 exists at the plasma membrane and on early endosome membranes in a GTP-bound form. The more efficient ADP-ribosylation of GTP-bound Rab5 (31), combined with our detection of more efficient Rab5 modification in the membrane fraction, are consistent with bacterially translocated ExoS preferentially modifying membrane associated Rab5-GTP, which interferes with activated Rab5 signaling processes.

TABLE 1

ExoS ADP-Ribosyltransferase Substrate Modification

Family	G-protein	Number of ADPR modifications *In vitro*	Number of ADPR modifications *In vivo*	Reference(s)
Ras	H-Ras	3	3	(13, 26)
	N-Ras	1	1	(13)
	K-Ras	1	1	(13)
	RalA/B	>2[a]	2	(18)
	Rap1	2	0[b]	(12, 32)
Rab	Rab3	+[c]	nd[d]	(10)
	Rab4	+	0	(10, 28)
	Rab5	3	2	(28, this study)
	Rab6	+	nd	(28)
	Rab7	+	+	(28, this study)
	Rab8	+	+	This study
	Rab11	3	3	This study
Rho	RhoA,B,C	nd	0	(10, this study)
	Rac	1	1	This study
	Cdc42	nd	+[e]	This study

[a] J. E. Fraylick, M. J. Riese, T. S. Vincent, J. T. Barbieri, and J. C. Olson (unpublished).
[b] Modification detected with transfected protein (16).
[c] Modified, but number of sites of ADP-ribosylation unknown.
[d] nd, not determined.
[e] Inefficient modification detected in membrane fraction.

Table 1 summarizes our current understanding of LMMG-protein substrate modification by ExoS. While this list does not recognize all potential *in vivo* substrates of ExoS ADPRT activity, 2DE proteomic analyses reveal a finite number of protein modifications in response to ExoS producing bacteria (McGuffie, Vincent, unpublished data). Further analyses of proteins

targeted by bacterially translocated ExoS ADPRT activity identified a hierarchical order of substrate modification within the cell. Ras and Ral are the first of the LMMG-proteins to be targeted by ExoS ADPRT activity, and alterations in their function by ExoS likely contribute to the effects of ExoS on cell growth. Multiple Rab proteins are targeted next, and alterations in their function by ExoS could explain effects of ExoS on microbial invasion and the regeneration of focal adhesion processes. Rac1 and Cdc42 modification requires a longer exposure to bacteria, and effects on their function by ExoS would be predicted to contribute to cytoskeletal alterations. Patterns of substrate modification track the translocation of ExoS from the membrane to the cytosol, and are consistent with the detection of approximately 2-fold higher levels of ExoS ADPRT activity in the membrane vs cytosolic fraction (11).

The complex interplay of LMMG-protein signal transduction pathways affected by ExoS complicates an understanding of the precise role of each substrate in the cellular effects of ExoS. Possibly more relevant to an understanding of the cellular mechanism of ExoS is the recognition of a progressive pattern of substrate modification, with the diverse and more severe effects of bacterially translocated ExoS on cell function relating to the coordinated effects of ExoS on the function of many LMMG-proteins. The integral role of the eukaryotic cell in directing substrate modification by bacterially translocated ExoS becomes evident in the more efficient ADP-ribosylation of membrane associated LMMG-protein substrates. The enhanced ADP-ribosylation of Rho and Rab family proteins as they recycle to the membrane also highlights the relationship between cellular activation and ExoS substrate availability.

ACKNOWLEDGMENTS

We thank Dara Frank for the bacterial strains used in this study. This work was supported by Public Heath Service Grants AI41694 and AI45569 from the National Institute of Allergy and Infectious Diseases and by the Medical University of South Carolina Institutional Research Funds.

REFERENCES

1. Yahr, T. L., Goranson, J., and Frank, D. W. (1996) Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Mol. Microbiol.* **22,** 991–1003.
2. Olson, J. C., McGuffie, E. M., and Frank, D. W. (1997) Effects of differential expression of the 49-kilodalton exoenzyme S by *Pseudomonas aeruginosa* on cultured eukaryotic cells. *Infect. Immun.* **65,** 248–256.
3. Olson, J. C., Fraylick, J. E., McGuffie, E. M., Dolan, K. M., Yahr, T. L., Frank, D. W., and Vincent, T. S. (1999) Interruption of multiple cellular processes in HT-29 epithelial cells by *Pseudomonas aeruginosa* exoenzyme S. *Infect. Immun.* **67,** 2847–2854.
4. Cowell, B. A., Chen, D. Y., Frank, D. W., Vallis, A. J., and Fleiszig, S. M. J. (2000) ExoT of cytotoxic *Pseudomonas aeruginosa* prevents uptake by corneal epithelial cells. *Infect. Immun.* **68,** 403–406.
5. Frithz-Lindsten, E., Du, Y., Rosqvist, R., and Forsberg, A. (1997) Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. *Mol. Microbiol.* **25,** 1125–1139.
6. Bruno, T. F., Buser, D. E., Syme, R. M., Woods, D. E., and Mody, C. H. (1998) *Pseudomonas aeruginosa* exoenzyme S is a mitogen but not a superantigen for human T lymphocytes. *Infect. Immun.* **66,** 3072–3079.
7. Goehring, U.-M., Schmidt, G., Pederson, K. J., Aktories, K., and Barbieri, J. T. (1999) The N-terminal domain of *Pseudomonas aeruginosa* exoenzyme S is a GTPase activating protein for Rho-GTPases. *J. Biol. Chem.* **274,** 36369–36372.
8. Knight, D. A., Finck-Barbancon, V., Kulich, S. M., and Barbieri, J. T. (1995) Functional domains of *Pseudomonas aeruginosa* exoenzyme S. *Infect. Immun.* **63,** 3182–3186.
9. Coburn, J., Wyatt, R. T., Iglewski, B. H., and Gill, D. M. (1989) Several GTP-binding proteins, including $p21^{c\text{-}H\text{-}ras}$, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. *J. Biol. Chem.* **264,** 9004–9008.
10. Coburn, J., and Gill, D. M. (1991) ADP-ribosylation of $p21^{ras}$ and related proteins by *Pseudomonas aeruginosa* exoenzyme S. *Infect. Immun.* **59,** 4259–4262.
11. Fraylick, J. E., La Rocque, J. R., Vincent, T. S., and Olson, J. C. (2001) Independent and coordinate effects of ADP-ribosyltransferase and GTPase-activating activities of exoenzyme S on HT-29 epithelial cell function. *Infect. Immun.,* 5318–5328.
12. McGuffie, E. M., Frank, D. W., Vincent, T. S., and Olson, J. C. (1998) Modification of Ras in eukaryotic cells by *Pseudomonas aeruginosa* exoenzyme S. *Infect. Immun.* **66,** 2607–2613.
13. Vincent, T. S., Fraylick, J. E., McGuffie, E. M., and Olson, J. C. (1999) ADP-ribosylation of oncogenic Ras proteins by *Pseudomonas aeruginosa* exoenzyme S *in vivo. Mol. Microbiol.* **32,** 1043–1053.
14. Ganesan, A. K., Vincent, T. S., Olson, J. C., and Barbieri, J. T. (1999) *Pseudomonas aeruginosa* exoenzyme S disrupts Ras-mediated signal transduction by inhibiting guanine nucleotide exchange factor catalyzed nucleotide exchange. *J. Biol. Chem.* **274,** 21823–21829.
15. Henriksson, M. L., Rosqvist, R., Telepnev, M., Wolf-Watz, H., and Hallberg, B. (2000) Ras effector pathway activation by epidermal growth factor is inhibited *in vivo* by exoenzyme S ADP-ribosylation of Ras. *Biochem. J.* **347,** 217–222.
16. Riese, M. J., Wittinghofer, A., and Barbieri, J. T. (2001) ADP-ribosylation of eukaryotic Rap1b by *P. aeruginosa* exoenzyme S. *In* 101th Annual Meeting American Society for Microbiology, pp. 108–109, Orlando, FL.
17. Fraylick, J. E., Vincent, T. S., and Olson, J. C. (2000) The role of Ral in the cellular mechanism of action of exoenzyme S. *In* 100th Annual Meeting American Society for Microbiology, pp. 67, Los Angeles, CA.
18. Fraylick, J. E., Vincent, T. S., and Olson, J. C. (2001) Characterization of the effects of exoenzyme S ADP-ribosyltransferase activity on RalA. *In* 101th Annual Meeting American Society for Microbiology, pp. 75, Orlando, FL.
19. Iglewski, B. H., Sadoff, J., Bjorn, M. J., and Maxwell, E. S. (1978) *Pseudomonas aeruginosa* exoenzyme S: An adenosine diphosphate ribosyltransferase distinct from toxin A. *Proc. Natl. Acad. Sci. USA* **75,** 3211–3215.
20. Kulich, S. M., Frank, D. W., and Barbieri, J. T. (1995) Expression of recombinant exoenzyme S of *Pseudomonas aeruginosa*. *Infect. Immun.* **63,** 1–8.
21. Pinto, M., Appay, M.-D., Simon-Assmann, P., Chevalier, G., Dra-

copoli, N., Fogh, J., and Zweibaum, A. (1982) Enterocyte differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. *Biol. Cell* **44,** 193–196.

22. Dolan, K. M., Lindenmayer, G., and Olson, J. C. (2000) Functional comparison of the NAD-binding cleft of ADP-ribosylating toxins. *Biochemistry* **39,** 8266–8275.
23. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227,** 680–685.
24. Antonny, B., Chardin, P., Roux, M., and Chabre, M. (1991) GTP hydrolysis mechanisms in ras p21 and in the ras–GAP complex studies by fluorescence measurements on the tryptophan mutants. *Biochemistry* **30,** 8287–8295.
25. Ganesan, A. K., Frank, D. W., Misra, R. P., Schmidt, G., and Barbieri, J. T. (1998) *Pseudomonas aeruginosa* exoenzyme S ADP-ribosylates Ras at multiple sites. *J. Biol. Chem.* **273,** 7332–7337.
26. Ganesan, A. K., Mende-Mueller, L., Selzer, J., and Barbieri, J. T. (1999) *Pseudomonas aeruginosa* exoenzyme S, a double ADP-ribosyltransferase, resembles vertebrate mono-ADP-ribosyltransferases. *J. Biol. Chem.* **274,** 9503–9508.
27. Huber, L. A. (1995) The mammalian two-dimensional gel map of small GTP-binding proteins. *In* Guidebook to the Small GTPases (Zerial, M., and Huber, L. A., Eds.), pp. 39–40, Oxford Univ. Press, New York.
28. Bette-Bobillo, P., Giro, P., Sainte-Marie, J., and Vidal, M. (1998) Exoenzyme S from *P. aeruginosa* ADP-ribosylates rab4 and inhibits transferrin recycling in SLO-permeabilized reticulocytes. *Biochem. Biophys. Res. Commun.* **244,** 336–341.
29. Takai, Y., Sasaki, T., and Matozaki, T. (2001) Small GTP-binding proteins. *Physiol. Rev.* **81,** 153–208.
30. Bos, J. G. (1998) All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO J.* **17,** 6776–6782.
31. Barbieri, A. M., Sha, Q., Bette-Bobillo, P., Stahl, P. D., and Vidal, M. (2001) ADP-ribosylation of Rab5 by ExoS of *Pseudomonas aeruginosa* affects endocytosis. *Infect. Immun.* **69,** 5329–5334.
32. Riese, M. J., Wittinghofer, A., and Barbieri, J. T. (2001) ADP-ribosylation of Arg42 of Rap by ExoS inhibits the ability of Rap to interact with its guanine nucleotide exchange factor, C3G. *Biochemistry* **40,** 3289–3294.