ADP-Ribosylation and Functional Effects of *Pseudomonas* Exoenzyme S on Cellular RalA[†]

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ABSTRACT: Exoenzyme S (ExoS) is a bifunctional virulence factor directly translocated into eukaryotic cells by the type III secretory process of *Pseudomonas aeruginosa*. Bacterial translocation of ExoS into epithelial cells is associated with diverse effects on cell function, including inhibition of growth, alterations in cell morphology, and effects on adherence processes. Preferred substrates of the ADP-ribosyltransferase (ADPRT) portion of ExoS include low molecular weight G-proteins (LMWG-proteins) in the Ras family. In examining the ADP-ribosylation and functional effects of ExoS on RalA, ExoS was found to ADP-ribosylate endogenous RalA and recombinant RalAΔCAAX at multiple sites, with Arg52 identified as the preferred site of ADP-ribosylation. The binding of RalA to the Ral binding domain (RBD) of its downstream effector, RalBP1, was inhibited by bacterially translocated ExoS, indicating an effect of ExoS on cellular RalA function. In vitro analyses confirmed that ADP-ribosylation of RalA directly interfered with its ability to bind to the RBD of RalBP1. The studies support the fact that RalA is a cellular substrate of bacterially translocated ExoS and that ADP-ribosylation by ExoS affects RalA interaction with its downstream effector, RalBP1.

The opportunistic pathogen *Pseudomonas aeruginosa* utilizes a contact-dependent type III secretory mechanism to deliver ExoS directly into susceptible eukaryotic cells. ExoS is a bifunctional molecule that contains an amino-terminal GTPase activating (GAP)[1] activity for LMWG-proteins of the Rho family (*1*) and a carboxy-terminal ADP-ribosyltransferase activity (*2*, *3*). In examining cellular targets of ExoS ADPRT activity in vitro, ExoS was found to transfer an ADP-ribose to many proteins in cell lysates, with LMWG-proteins in the Ras superfamily, including Ras, Ral, Rap1, Rab1, Rab3, and Rab4, being preferred substrates (*4*, *5*). Utilizing a bacterial eukaryotic cell coculture system to examine cellular proteins targeted by bacterially translocated ExoS, Ras was confirmed as a substrate of ExoS ADPRT activity within intact cells, whereas endogenous Rap1 was not, indicating ExoS substrate selectivity within the cell (*6*, *7*). In coculture studies comparing the effects of the ExoS-producing *P. aeruginosa* strain, 388, and its isogenic mutant, 388ΔS (*8*), on HT-29 epithelial cells, ExoS production was associated with interference of cell proliferation, severe effects on cell morphology, effacement of cell surface structures, and loss of re-adherence processes (*9*). Subsequent studies examining the effects of *P. aeruginosa* strains producing ADPRT active or inactive forms of ExoS confirmed the requirement for ExoS ADPRT activity for severe effects of ExoS on cell growth and morphology (*10*). This directed attention to cellular proteins targeted by ExoS ADPRT activity, such as Ras, in mediating effects of ExoS on cell function.

In examining the effects of ExoS on Ras function, in vitro and bacterial translocation analyses confirmed the ADP-ribosylation of H-Ras at multiple sites, with the preferred site of ADP-ribosylation at Arg41 (*11*, *12*). Consistent with the ADP-ribosylation of Ras contributing to the cellular effects of bacterially translocated ExoS, the ADP-ribosylation of Ras at Arg41 inhibited its guanine nucleotide exchange factor (GEF) mediated nucleotide exchange in vitro and the association of Ras with its downstream effector, Raf-1, within cells (*12*−*14*). Since Ras plays a central role in multiple cell signaling processes, the ADP-ribosylation of Ras alone could account for the diverse effects of ExoS on cell function. However, the ability of ExoS to ADP-ribosylate multiple LMWG-proteins in vitro implicated the potential for ExoS to affect the function of other LMWG-proteins within the cell.

The in vitro substrate of ExoS, Ral, is a ubiquitously expressed member of the Ras family of LMWG-proteins. Ral is posttranslationally geranylgeranylated and almost exclusively associated with cell membranes, including the

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[1] Abbreviations: ADPRT, ADP-ribosyltransferase; BSA, bovine serum albumin; CFU, colony-forming units; ECL, enhanced chemiluminescence; 2DE, two-dimensional electrophoresis; FBS, fetal bovine serum; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GSH, glutathione; GST−RBD, glutathione *S*-transferase−Ral binding domain fusion probe; LMWG-protein, low molecular weight G-protein; p*I*, isoelectric point; PLD, phospholipase D; RalBP1, Ral binding protein 1; RBD, Ral binding domain; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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plasma membrane, endocytic vesicles, synaptic vesicles, and specialized secretory organelles (*15*, *16*). Properties of endogenous Ral have complicated analyses of its function, and as a consequence it has proven to be difficult to gain a precise understanding of the role of Ral within the cell. Ral can be activated by diverse extracellular stimuli (*17*) and lies downstream of Ras in cell signaling pathways (*18*). The downstream effect of Ras on Ral function was confirmed when activated Ras was shown to bind RalGDS, a Ral GEF, recruiting RalGDS to the plasma membrane where it catalyzed Ral activation (*19*, *20*). Ral proteins have been found to participate in the activation of multiple cellular proteins. Active, GTP-bound Ral associates with Ral binding protein 1 (RalBP1) (*15*), filamin (*21*), and Sec5 (*22*, *23*). Ral is also able to activate in a nucleotide-independent manner phospholipase D (PLD) (*24*), the LMWG-protein, Arf (*25*), Src kinase (*26*), and the transcription factor, NF-κB (*27*). Ral has been implicated as a regulator of filopodia formation through its interaction with filamin A, an actin cross-linking protein (*21*), and Sec5, an integral component of the exocyst complex involved in targeting vesicles to sites of secretion (*22*, *23*). RalBP1, a GAP for Rac and Cdc42 (*15*), links Ral activation to actin cytoskeletal remodeling, while RalPB1 binding to POB1 and Reps1 links Ral to endocytic processes (*28*, *29*). Stimulation of the Ras−Ral pathway results in the RalA-dependent activation of PLD, a regulator of phospholipid metabolism implicated in vesicular trafficking (*15*, *24*). The ability of dominant negative Ral to block Ras transformation also identifies a role of Ral in Ras-mediated transformation (*30*). Together, these studies implicate the involvement of Ral in endocytosis and vesicular transport, Cdc42-mediated filopodia formation, cell growth, and cell transformation (*31*).

Ral is recognized as a candidate substrate of bacterially translocated ExoS on the basis of its ability to serve as a substrate of ExoS ADPRT activity in vitro, its similarity to Ras, and its function in pathways governing cellular proliferation and cytoskeletal regulation, known to be affected by ExoS. The studies described herein confirm that endogenous RalA is a substrate of bacterially translocated ExoS ADPRT activity and identify Arg52 as a preferred site of modification. The ADP-ribosylation of RalA was also shown to alter its ability to bind its downstream effector, RalBP1, independent of effects of ExoS on Ras function.

EXPERIMENTAL PROCEDURES

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 (*2*) and strain 388Δ*exoS* (388ΔS), an isogenic mutant that lacks production of ExoS (*8*). Bacterial strains were grown in ExoS induction medium for 16 h in preparation for coculture with eukaryotic cells, as previously described (*32*). Bacteria were then diluted on the basis of culture OD_{590} to approximately 10^7 CFU/mL in tissue culture medium containing 0.6% bovine serum albumin (BSA) (Sigma, St. Louis, MO) and added to eukaryotic cells at a multiplicity of infection (MOI) of 50.

Eukaryotic Cell Culture. HT-29 colon carcinoma cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were maintained at 37 °C in 5% CO_2−95% air in McCoy's 5A medium, containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco-BRL, Rockville, MD) (McCoy's FBS). In preparation for culture with bacteria, HT-29 cells were detached with 0.05% trypsin−0.53 mM EDTA (trypsin−EDTA) (Gibco-BRL) and seeded in culture dishes (Costar, Cambridge, MA). After 48 h, cells at 45−50% confluency were cultured alone or with bacteria for 4−6 h in McCoy's BSA and monitored for RalA modification and effects on RalA function.

Analysis of Cellular Ral ADP-Ribosylation by Bacterially Translocated ExoS. (i) SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE) Analysis of Ral Modification. ADP-ribosylation of endogenous Ral by ExoS was initially assessed on the basis of an alteration in protein mobility by SDS−PAGE following exposure of eukaryotic cells to ExoS producing strain 388 or 388ΔS, as previously described for Ras (*6*). After exposure to bacteria, cells were lysed in Laemmli electrophoresis sample buffer (*33*), heated to 95 °C for 3 min, resolved by 12% SDS−PAGE, and immunoblotted. RalA was detected using mouse monoclonal anti-RalA antibody (BD Transduction Laboratories, San Diego, CA), followed by peroxidase conjugated anti-mouse IgG (Sigma), and visualized using enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL).

(ii) Two-Dimensional Electrophoresis (2DE) Analysis of Ral Modification. For 2DE analysis, eukaryotic cells were lysed following coculture with bacteria in 2DE lysis buffer [8 M urea, 2% Triton X-100, 0.3% dithiothreitol, 1.5% Pharmalytes (Pharmacia, Piscataway, NJ)] for 30 min on ice. Samples in 2DE lysis buffer were used to rehydrate immobilized pH gradient gel strips (7 cm, pH 3−10; Pharmacia) overnight. Proteins were focused on the basis of the isoelectric point (p*I*) for 24 h at 3500 V. Following isoelectric focusing, strips were equilibrated in SDS equilibration buffer (50 mM Tris, pH 6.8, 6 M urea, 30% glycerol, 2% SDS) containing 20 mg/mL dithiothreitol for 10 min and then equilibrated in SDS equilibration buffer containing 25 mg/mL iodoacetamide for 10 min. Proteins were resolved in the second dimension by 12% SDS−PAGE, transferred to PVDF membranes (Millipore, Bedford, MA), and immunoblotted for RalA as described above. An internal control (LA059 monoclonal antibody; Quality Biotech, Camden, NJ) was included with each sample to allow the alignment of Ral isoforms.

Analysis of Ral ADP-Ribosylation by ExoS in Vitro. (i) Construction of the RalA Clone. A construct encoding simian RalA, a kind gift of Armand Tavitian (UNSERM, Paris, France), was used to generate a PCR fragment containing RalA that was cloned into pET15b to permit production of recombinant His_6RalA. To produce RalAΔCAAX and RalA R52KΔCAAX, RalA was subcloned into M13mp19 and subjected to Scultptor mutagenesis as described by the manufacturer (Amersham-Pharmacia).

(ii) Analysis of RalA ADP-Ribosylation in Vitro. For SDS−PAGE analyses of endogenous RalA ADP-ribosylation by purified ExoS in vitro, HT-29 cells were lysed on ice in TBS−TDS (10 mM Tris, pH 7.4, 140 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS), and 50 μL of lysate was incubated with 0.2 μM ExoS, 0.2 μM 14-3-3, 10 mM NAD, and 1 mM $MgCl_2$ in 0.2 M Tris−acetate, pH 6.0, for 1 h at 25 °C. Reactions were stopped by the addition

of 4× Laemmli electrophoresis sample buffer and resolved by SDS−PAGE and analyzed for RalA as above. RalΔCAAX, which lacks the four C-terminal amino acids, was used in all other in vitro ExoS ADPRT reactions because of difficulties in analyzing endogenous RalA. For 2DE analyses, 5 μM His_6RalAΔCAAX was ADP-ribosylated by purified ExoS in vitro as described above, samples were cup-loaded onto pH 3−10 Immobiline dry strips and focused, and RalA was detected as in 2DE analyses (see above). For biochemical analyses of ADP-ribosylated RalA, ExoS ADPRT reactions were performed essentially as previously described (*7*) using 5 μM His_6RalAΔCAAX or His_6RalA R52KΔCAAX, 0.1 μM 14-3-3 protein, 0.1 mM [adenylate-phosphate-^{32}P]NAD, and 3 μM BSA in 50 mM Tris-HCl, pH 8.0, in the presence of 4 nM ExoS (linear velocity determinations) or 16 nM ExoS (stoichiometry determinations). Reactions were stopped at 60 min, or as indicated, by the addition of one-half volume of Laemmli electrophoresis sample buffer and then subjected to SDS−PAGE, and incorporation of radiolabel was quantified from excised gel pieces. In reactions intended for MALDI-TOF, reaction mixtures did not contain BSA and were stopped with an equal volume of 20% TCA. Mixtures were then incubated at 4 °C for 1 h before centrifugation, and pellets were washed twice with an equal volume of ice-cold acetone and air-dried.

(iii) MALDI-TOF Analysis of RalA from in Vitro Experiments. For analysis of full-length RalA, 500 pmol of TCA-precipitated RalA was suspended in 1.5 μL of 0.1% TFA−60% acetonitrile saturated with sinapinic acid. Resuspended RalA was applied directly to a 96-well MALDI-TOF plate for analysis. To examine digested RalA, 250 pmol of modified or unmodified RalA was proteolyzed overnight at 37 °C with 3 μg of V8 protease in 2 M urea and 100 mM sodium phosphate, pH 7.8. Samples were subjected to SDS−PAGE to ensure ADP-ribosylation and proteolytic digestion. Samples were subsequently lyophilized and suspended in 25 μL of 0.1% TFA, and 2.5 μL was bound to Zip-Tip C_{18} resin (Millipore) and eluted into 0.1% TFA−60% actetonitrile saturated with sinapinic acid and dried on a MALDI-TOF plate. APE Biosystems Voyager-DE/PRO was used for MALDI-TOF analysis. Predictions of peptide masses were made using MS-Digest at ProteinProspector (UCSF). ADP-ribosylation of peptides and proteins was calculated to result in a 541.1 Da increase in monoisotopic mass (peptides) and a 541.3 Da increase in average mass (full-length proteins).

Analysis of Effects of ADP-Ribosylation by ExoS on RalA Function. (i) Construction of the Ral Binding Domain (RBD)−Glutathione S-Transferase (GST) Fusion Probe. The RBD sequence, encoding amino acids 397−518, of the downstream effector of Ral, Ral binding protein 1 (RalBP1), was amplified from a cDNA library. The forward primer, 5′-CGCGGATCCGGCATCAAAGGAGGAGATCAG-3′, and reverse primer, 5′-CGGGAATTCTCTGAGGCGTTCAA-TCTCTTC-3′, included *Bam*HI and *Eco*RI restriction sites, respectively. The primers amplified a 384 bp product, which was excised from a 1% agarose gel, digested with *Bam*HI and *Eco*RI, and ligated into the pGEX-4T-1 vector (Pharmacia) for expression as a GST fusion protein (GST−RBD). The vector containing the GST−RBD fusion construct was electroporated into *Escherichia coli* strain BL21 electrocompetent cells, and transformed cells were selected for ampicillin resistance (100 μg/mL). The gene encoding GST−RBD was sequenced to confirm the correct insertion and the absence of aberrations. Cells expressing the GST−RBD fusion protein were induced with 0.1 mM IPTG for 2 h, then centrifuged at 10000*g* for 10 min, resuspended in 10 mL of B-PER bacterial protein extraction reagent (Pierce, Rockford, IL), and lysed at room temperature for 10 min. Bacterial lysate containing GST−RBD was added to glutathione (GSH)−Sepharose beads, washed, and resuspended in PBS to make a 50% slurry. The concentration of GST−RBD bound to GSH was quantified by 12% SDS−PAGE, using soybean trypsin inhibitor as a protein standard.

(ii) GST Pull Down of GTP Active RalA. To assess the activational state of RalA following its modification by bacterially translocated ExoS, HT-29 cells were cocultured with strain 388 or 388ΔS for 6 h and lysed for 20 min on ice in RBD pull-down lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 1% Nonidet P-40, 1 mM PMSF, 10 μg/mL each leupeptin and aprotinin (Sigma)] as previously described (*34*). Lysates were centrifuged at 16000*g* for 2 min to remove cellular debris and unbroken cells, and the supernatant was incubated with 8 μg of GST−RBD bound to GSH beads for 2 h at 4 °C with rocking. The beads were then washed four times with lysis buffer, resuspended in Laemmli electrophoresis sample buffer, resolved by 12% SDS−PAGE, and transferred to PVDF membranes, and RalA was immunoblotted as described above. To assess the ability of ADP-ribosylated RalA to bind RalBP1, RalA was ADP-ribosylated by purified ExoS in an in vitro reaction, as previously described (*12*), using 5 μM His_6RalAΔCAAX loaded with GDP or GTPγS according to the procedure of Antony et al. (*35*). Reaction mixtures were then incubated with 10 μg of GST−RBD bound to GSH beads, processed, and visualized as described above.

RESULTS

Modification of RalA by ExoS. To initially assess whether cellular RalA was a substrate of bacterially translocated ExoS, endogenous RalA was examined for a shift in molecular mass by SDS−PAGE following coculture of HT-29 epithelial cells with ExoS-producing *P. aeruginosa*. A shift in mass following exposure to ExoS-producing strain 388, but not the isogeninc non-ExoS-producing strain 388ΔS, is indicative of RalA ADP-ribosylation by bacterially translocated ExoS. RalA from HT-29 cells treated with 388ΔS resolved at a mass of ∼28 kDa (Figure 1a), and appeared identical to that of untreated control cells (not shown). Upon treatment of HT-29 cells with ExoS-producing strain 388, endogenous Ral showed two shifts in molecular mass, consistent with at least two sites of modification by bacterially translocated ExoS. To further support the fact that the shifts in mass of endogenous RalA related to its ADP-ribosylation by ExoS, in vitro ADPRT reactions were performed on endogenous RalA in HT-29 cell extracts using purified ExoS. As shown in Figure 1a, RalA exhibited a shift in mass following an in vitro ADPRT reaction; however, the shift was more efficient than that detected by bacterially translocated ExoS. Modifications detected in RalA by SDS−PAGE following coculture with ExoS-producing bacteria and in vitro ExoS ADPRT reactions are consistent with RalA being a substrate of bacterially translocated ExoS ADPRT activity.

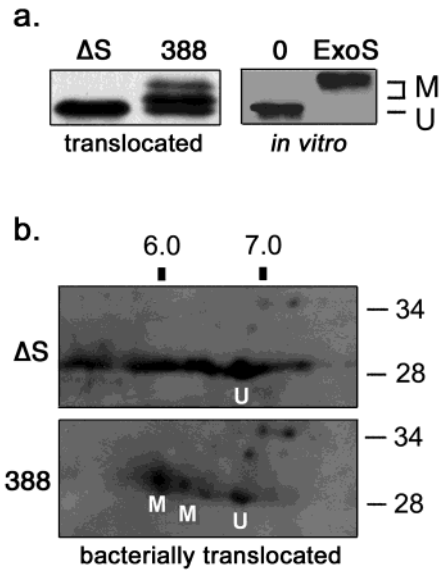

FIGURE 1: Analysis of modification of endogenous RalA by ExoS. (a) SDS−PAGE analysis of RalA modification. Translocated: HT-29 epithelial cells were cocultured with strains 388 or 388ΔS (ΔS) for 5 h. HT-29 cells were lysed in Laemmli sample buffer, separated by 12% SDS−PAGE, transferred to a PVDF membrane, probed with a RalA specific antibody, followed by HRP conjugated anti-mouse IgG, and developed using ECL. In vitro: HT-29 cells were lysed on ice in TBS−TDS, and 50 μL of lysate was incubated with 0.2 μM ExoS, 0.2 μM 14-3-3, 10 mM NAD, and 1 mM $MgCl_2$ in 0.2 M Tris−acetate, pH 6.0, for 1 h at 25 °C. RalA was resolved and analyzed as above. (b) 2DE analysis of RalA modification by bacterially translocated ExoS. HT-29 cells were cocultured with bacteria as in (a) and lysed in 2DE lysis buffer for 30 min on ice. The reactions were used to rehydrate Immobiline dry strips (pH 3−10), and Ral was focused in the first dimension on the basis of p*I*. Strips were then equilibrated in SDS equilibration buffer, and proteins were resolved in the second dimension by 12% SDS−PAGE. RalA was detected as above. Protein mass is indicated to the right and p*I* above. The data are representative of multiple independent experiments, and unmodified (U) and modified (M) forms of RalA are labeled.

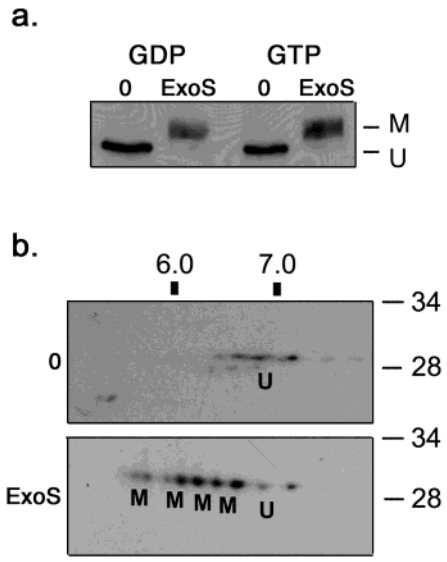

FIGURE 2: Analysis of modification of recombinant His_6RalA-ΔCAAX by ExoS in vitro. (a) SDS−PAGE analyses. 5 μM GDP or GTP-loaded RalAΔCAAX was modified in an in vitro ExoS ADPRT reaction as described above, and RalA was detected as in Figure 1a. (b) 2DE analyses. RalAΔCAAX was modified by ExoS in an in vitro reaction, and samples were cup-loaded onto pH 3−10 Immobiline dry strips, then resolved as in Figure 1b, blotted, and probed for RalA. Mass is indicated to the right of the image and p*I* above. Positions of unmodified (U) and modified (M) Ral are labeled, and the data shown are representative of multiple independent experiments.

2DE Analysis of ExoS Modification of Ral by Bacterially Translocated ExoS. It has proven to be experimentally difficult to confirm the ADP-ribosylation of cellular proteins by bacterially translocated ExoS while retaining cell membrane integrity. Cells are impermeable to NAD, the source of ADP-ribose in the ExoS ADPRT reaction, which precludes the direct use of radiolabeled NAD to identify ADP-ribosylated substrates within cells. Also, unlike phosphorylated proteins, there are currently no commercially available antibodies to detect ADP-ribosylated proteins. Two-dimensional electrophoresis, which resolves proteins on the basis of mass and p*I*, provides an alternative method for assessing the ADP-ribosylation of cellular proteins. This method was previously used to confirm the ADP-ribosylation of cellular H-Ras at three sites by bacterially translocated ExoS (*12*), identified in in vitro reactions as Arg41, Arg128, and Arg135 (*11*, *36*).

In 2DE analysis of unmodified, endogenous RalA from nonbacterial or 388ΔS-treated HT-29 cells, RalA was found to be more difficult to focus than that previously observed for Ras, resolving as a series of 28 kDa spots having a focal p*I* of ∼6.8. Difficulties in resolving RalA were presumed to relate to its posttranslational geranylgeranylation-linked membrane association (*37*). Following exposure of HT-29 cells to ExoS-producing bacteria, the RalA isoform group exhibited what was interpreted as two or three shifts in mass and p*I* (from a p*I* of ∼6.8 to 6.5, then to 6.2, and then to 6.0), highly similar to that previously observed upon the ADP-ribosylation of cellular H-Ras (*12*). The shift in RalA detected by 2DE is consistent with the addition of two to three negatively charged ADP-ribose moieties by bacterially translocated ExoS.

Analysis of ExoS ADP-Ribosylation of RalAΔCAAX. To allow biochemical analyses of the ADP-ribosylation of RalA by ExoS, the C-terminal CAAL membrane-binding motif of His_6RalA was genetically deleted, producing RalAΔCAAX, which was more amenable to in vitro analyses. RalAΔCAAX exhibited a shift in mass by SDS−PAGE following an in vitro ExoS ADPRT reaction similar to that of ADP-ribosylated endogenous RalA (Figure 2a). RalAΔCAAX also appeared to be modified efficiently in its GTP- or GDP-bound form, indicating that, like Ras (*12*, *36*), both active GTP and inactive GDP RalA can be ADP-ribosylated by ExoS. Unmodified RalAΔCAAX proved easier to resolve by 2DE than endogenous RalA, focusing as three to four distinct spots at a p*I* of ∼6.9, its slightly higher p*I* reflecting the histidine residues added for purification (Figure 2b). 2DE analysis of RalAΔCAAX ADP-ribosylation by ExoS in vitro identified multiple shifts in isoform groups, which were interpreted as four potential sites of ADP-ribosylation. The detection of more efficient ADP-ribosylation of endogenous RalA or RalAΔCAAX in in vitro ADPRT reactions suggests that the cellular environment, and possibly the CAAX sequence, influences the accessibility of arginine residues in RalA to ADP-ribosylation by bacterially translocated ExoS.

Analysis of Residues in RalA ADP-Ribosylated by ExoS. In vitro analyses have confirmed that Arg41 in H-Ras and

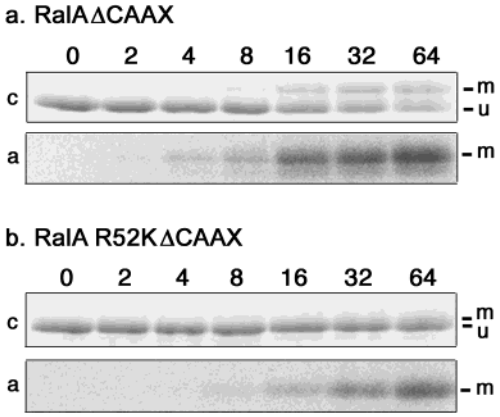

FIGURE 3: ExoS ADP-ribosylates RalA at Arg52. (a) 5 μM RalAΔCAAX or (b) 5 μM RalA R52KΔCAAX was incubated with 0.1 μM 14-3-3, 0.1 mM [^{32}P]NAD, and 3 μM BSA in 50 mM Tris-HCl, pH 8.0, in the absence (0) or presence of 4 nM ExoS for the indicated time (in minutes). Reactions were stopped by the addition of Laemmli electrophoresis sample buffer and subjected to SDS−PAGE. Coomassie Blue stained panels (c) and panels exposed to X-ray film (a) are labeled. The incorporation of radiolabel is quantified in Table 1.

Table 1: ADP-Ribosylation of RalA by ExoS in Vitro

	ADP-ribosyltransferase reaction	
protein	stoichiometry[a] [mol of NAD (mol of Ral)$^{-1}$]	linear velocity[b] [mol of NAD min^{-1} (mol of ExoS)$^{-1}$]
RalAΔCAAX	2.15 ± 0.27	3.33 ± 0.33
RalA R52KΔCAAX	1.93 ± 0.16	0.98 ± 0.17

[a] Stoichiometry was determined from 20 μL reactions containing 5 μM His$_6$RalAΔCAAX or His$_6$RalA R52KΔCAAX, 16 nM ExoS (233−453), 3 μM BSA, 0.1 mM [^{32}P]NAD, and 0.1 μM 14-3-3 in 50 mM Tris-HCl (pH 8.0). The reaction was incubated at room temperature for 60 min and stopped by the addition of Laemmli electrophoresis sample buffer. The samples were resolved by SDS−PAGE and stained with Coomassie Blue. Protein bands were excised, and radioactivity was measured in a scintillation counter. [b] Linear velocity was measured as described in Figure 3. Values are an average of at least two independent experiments.

Rap1b are preferred sites of ADP-ribosylation by ExoS (*7*, *11*). The transfer of an ADP-ribose moiety to this residue has also been associated with a large, 3 kDa shift in apparent molecular mass, characteristic of the ADP-ribosylation of certain LMWG-proteins by ExoS (*4*). To determine if the Arg41 homologue in RalA, Arg52, is a site of modification by ExoS, RalAΔCAAX and RalA R52KΔCAAX ADP-ribosylation were compared in an in vitro reaction using purified ExoS. The incubation of ExoS with RalA resulted in a time-dependent large shift in mass characteristic of ADP-ribosylation by ExoS (Figure 3). In comparison, a small shift in mass was evident upon treatment of RalA R52K with ExoS, which corresponded with the incorporation of radiolabeled ADP-ribose. In linear velocity reactions, the ADP-ribosylation of RalA by ExoS was determined to be three times greater than that of RalA R52K (Table 1). The increased rate of incorporation of ADP-ribose into RalA, as compared to RalA R52K, is supportive of Arg52 being a preferred site of modification by ExoS. The large shift in mass detected upon the ADP-ribosylation of RalA at Arg52 also supports the fact that this residue functions in a manner similar to that of Arg41 of Ras (*4*, *11*), in introducing alterations in protein structure when ADP-ribosylated by ExoS.

Table 2: MALDI-TOF of RalA and ADP-Ribosylated RalA[a]

protein	predicted mass	obsd mass (±SD)	estimated no. of ADP-ribosylations
RalAΔCAAX	25298	25310 ± 5	
ADP-ribosylated RalAΔCAAX	25298	25312 ± 7	
	25840	NO[b]	1
	26380	26410 ± 1	2
	26921	26922 ± 7	3
	27463	27461 ± 9	4
	28004	27997 ± 5	5
RalA R52KΔCAAX	25270	25295 ± 28	
ADP-ribosylated RalA R52KΔCAAX	25270	NO	
	25811	NO	1
	26352	26382 ± 18	2
	26894	26904 ± 23	3
	27435	27432 ± 20	4

[a] His$_6$RalA and His$_6$RalA R52K were treated as described for the stoichiometry reactions in Table 1, except that BSA and sample buffer were not added. Unmodified or modified RalA (500 pmol) was precipitated with TCA, washed twice with acetone, and resuspended in 0.1% TFA−60% acetonitrile saturated with sinapinnic acid. Mixtures were applied to MALDI-TOF plates and subjected to 10−100 shot ionizations from different regions of the dried matrix. Control mixtures containing 14-3-3 and ExoS, but not RalA, did not exhibit any of the peaks reported above. Only clearly resolved peaks with intensity at least 50% of the base peak were considered. For each reported peak, a smaller, secondary peak of approximately 180 Da was observed. The reported values and standard deviations (SD) are from two separate MALDI-TOF analyses of two independent experiments. [b] NO, not observed.

ADP-ribosylation reactions carried to completion revealed that approximately 2 mol of ADP-ribose were incorporated per mole of RalA or RalA R52K (Table 1), implying that ExoS modified both forms of RalA most efficiently at two sites. To further examine RalA ADP-ribosylation by ExoS, unmodified and modified RalA were subjected to MALDI-TOF mass spectrometry. While unmodified RalA was found to be similar in mass to that predicted from its amino acid composition, ADP-ribosylated RalA was found to be present as a mixture of proteins which included unmodified RalA and RalA modified two, three, four, or five times (Table 2). Mass determination of ADP-ribosylated RalA R52K revealed forms with two, three, and four modifications (Table 2). These results are consistent with 2DE analyses that identified multiple sites of ADP-ribosylation on RalAΔCAAX by ExoS in vitro and also supported the fact that Arg52 was a site of modification. MALDI-TOF analysis of V8 proteolytic digests of modified and unmodified RalA produced several spectral peaks unique to modified RalA. These included peaks at 1384.7 ± 0.7 and 1603.5 ± 0.3 Da, which are similar to peaks expected from V8 digests of RalA modified at Arg135 (1386.5 Da) and at Arg161 (1603.6 Da). Together, the data support the fact that Arg52 functions as a preferred site of ADP-ribosylation in vitro and that Arg135 and Arg161 function as secondary sites of ADP-ribosylation by ExoS.

Effects of ExoS ADP-Ribosylation on RalA Activation. To determine if the ADP-ribosylation of RalA by ExoS affects its cellular function, the activation state of RalA was examined using the RalA binding domain (RBD) of RalBP1 fused to GST to detect GTP-bound RalA (*34*). Active, GTP-bound RalA was then preferentially pulled down using

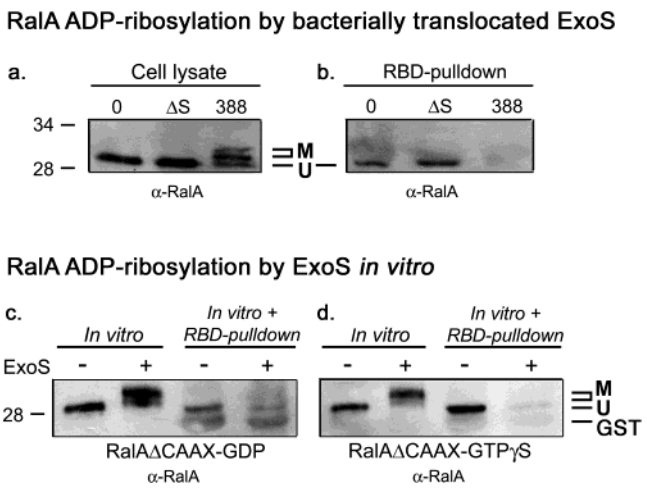

FIGURE 4: Inhibition of RalA activation by bacterially translocated ExoS. Bacterially translocated ExoS: HT-29 cells in six-well culture plates were cocultured with 1×10^7 CFU/mL 388 or 388ΔS (ΔS) or no bacteria (0) for 6 h. To examine RalA activation, cells were washed with PBS after removal of bacteria and lysed in 250 μL of pull-down lysis buffer on ice for 20 min. Cell lysates from two wells (500 μL total volume) were pooled, and an aliquot was reserved for SDS−PAGE analysis (a). The remainder of the lysate was incubated with 8 μg of GST−RBD bound to GSH beads for 2 h at 4 °C to pull down active RalA−GTP (b). In vitro: RalAΔCAAX (70 ng) was loaded with GDP (c) or GTPγS (d) and ADP-ribosylated by ExoS, or not, in an in vitro reaction. The reaction mixture was then incubated for 2 h at 4 °C with 10 μg of GST−RBD, and active RalA−GTP was isolated as in (b). All samples were separated by 12% SDS−PAGE and blotted onto PVDF membranes, and RalA was detected as in Figure 1a. Modified (M) and unmodified (U) RalA and coprecipitated GST are labeled. The results are representative of four independent experiments.

GST−RBD bound to GSH beads and detected by immunoblotting with anti-RalA antibody.

In examining the effects of ExoS on activation of endogenous RalA following treatment of HT-29 cells with ExoS producing strain 388, two shifts in the molecular mass of RalA were detected (Figure 4a), as previously recognized following the ADP-ribosylation of cellular RalA by bacterially translocated ExoS (refer to Figure 1a). When the activational state of the ADP-ribosylated RalA was analyzed on the basis of its binding to GST−RBD, minimal active RalA was detected in either its modified or apparently unmodified forms (Figure 4b). The inability to detect active, unmodified RalA following bacterial translocation of ExoS suggested that alternative mechanisms, other than the direct ADP-ribosylation of RalA, might be affecting RalA activation within the cell. In comparison, active, unmodified RalA was detected in control cells and cells treated with the non-ExoS-producing strain 388ΔS.

RalA is downstream of Ras in the cell signaling network. Since Ras is also a substrate of bacterially translocated ExoS, and ExoS is known to interfere with Ras activation (*6*, *12*, *13*), it is possible that ExoS might indirectly affect cellular RalA activation through interference of Ras activation, and/or directly affect RalA activation through its ADP-ribosylation. To determine whether the ADP-ribosylation of RalA by ExoS can directly affect RalA function, binding of purified His_6RalAΔCAAX to the RBD of RalBP1 was assayed following its ADP-ribosylation in vitro by purified ExoS. Comparisons of RalBP1 binding to RalAΔCAAX loaded with GDP or GTPγS are shown in Figure 4c,d. The first two lanes in this figure compare the input products, ADP-ribosylated or not by ExoS in vitro, and the last two lanes show GST−RBD pull-down assays. The preferential binding of RalBP1 to active GTP-bound RalA is evident in comparisons of the two panels. However, limited binding of GDP-loaded RalA to RalBP1 was also apparent in these analyses, similar to the preferred but not exclusive binding previously observed for the interaction of activated Ras with the CRD-Ras binding domain of Raf-1 (*38*, *39*). In analyses of the binding of GTP-activated RalA to GST−RBD, the ADP-ribosylation of RalAΔCAAX GTPγS by ExoS was found to effectively inhibit binding to the downstream effector. The interference of RalA−GTP binding to its effector, RalBP1, following its ADP-ribosylation by ExoS, indicates that ExoS is able to directly affect RalA function, independently of upstream effects of ExoS on Ras function. In relating effects of ExoS on RalA binding to RalBP1 in vitro to that observed following bacterial translocation of ExoS, the data support that direct, as well as indirect, effects of ExoS upstream of RalA likely contribute to the interference of RalA binding to RalBP1 observed following exposure to ExoS-producing bacteria.

DISCUSSION

With the recognition of the role of ExoS ADPRT activity in effects of ExoS on cell function (*10*), it became apparent that understanding the endogenous, cellular substrate specificity of ExoS ADPRT activity was integral to determining the mechanism for the effects of ExoS. Initial studies confirmed that cellular Ras was ADP-ribosylated by bacterially translocated ExoS, while its closely related family member, Rap1, appeared not to be an efficient substrate within the cell (*6*, *7*). Ral, another Ras family protein, is also a substrate of ExoS ADPRT activity in vitro (*5*) and, as such, a potential target of ExoS ADPRT activity when bacterially translocated. We therefore chose to explore the efficiency of Ral ADP-ribosylation by bacterially translocated ExoS and examine how its modification by ExoS might contribute to the effects of ExoS on eukaryotic cell function.

LMWG-proteins can be screened as possible cellular targets of bacterially translocated ExoS ADPRT activity on the basis of a detectable shift in mass following exposure to ExoS-producing bacteria. Two shifts in the molecular mass of RalA were detected following the coculture of HT-29 epithelial cells with an ExoS-producing *P. aeruginosa* strain, which was consistent with cellular RalA being a substrate of bacterially translocated ExoS and having at least two sites of ADP-ribosylation. 2DE analyses confirmed that RalA exhibited shifts in mass and p*I* following exposure to ExoS-producing bacteria highly similar to that previously observed for ADP-ribosylated H-Ras (*12*) and supported the ADP-ribosylation of RalA at two or three sites by bacterially translocated ExoS.

Since it proved difficult to determine the molecular basis of RalA modification by ExoS using endogenous RalA, a C-terminal CAAX deletion mutant of RalA was constructed to allow further characterization of RalA ADP-ribosylation by ExoS. Stoichiometry analyses of RalAΔCAAX revealed that 2.15 mol of ADP-ribose was incorporated per mole of RalA, which was consistent with the detection of two to three sites of ADP-ribosylation of cellular RalA by bacterially translocated ExoS. MALDI-TOF mass spectrometry analyses

of in vitro ExoS ADPRT reactions found ExoS to modify RalAΔCAAX 2−5 times, which was corroborated by 2DE analyses. Notable in comparisons of the ADP-ribosylation of RalAΔCAAX in vitro and that following bacterial translocation of ExoS was the general increased efficiency of RalA modification detected in vitro. These comparisons indicate that the cellular association of RalA and/or the mode of internalization of ExoS limit the efficiency of RalA modification within the cell. Ral shares a high degree of amino acid sequence homology with Ras (50−55%) and includes an arginine residue at position 52 that is analogous to Arg41 in Ras, the preferred site of ADP-ribosylation by ExoS (*11*). Comparisons of the stoichiometry and estimated number of ADP-ribosylations of RalAΔCAAX and RalA R52KΔCAAX are consistent with Arg52 being a site of ADP-ribosylation by ExoS. The 3-fold decrease in the velocity of ADP-ribosylation of RalA upon the introduction of the R52K mutation also supports the fact that Arg52 is a preferred site of ADP-ribosylation by ExoS based on in vitro analyses. In addition to Arg52, spectral peaks were identified that correspond to modifications of RalA at Arg135 and Arg161 by ExoS, supporting the fact that these residues are favored secondary sites of ADP-ribosylation. While up to five sites of Ral ADP-ribosylation can be detected in vitro, our studies support the fact that RalA is modified by ExoS most efficiently at two sites. The large shift in mass observed following bacterial translocation of ExoS supports the fact that Arg52 also functions as a site of ADP-ribosylation within the cell. However, it remains to be determined whether Arg52 represents the preferred site of ADP-ribosylation by ExoS within the cell.

In vitro analyses found the ADP-ribosylation of H-Ras and Rap1 by ExoS to interfere with their GEF-catalyzed GTP exchange (*7*, *13*). In examining how the ADP-ribosylation of RalA by ExoS might affect its function, bacterially translocated ExoS was found to interfere with endogenous RalA activation, as assessed by interference of its binding to the downstream effector RalBP1. Ral lies downstream of Ras in cell signal transduction pathways affecting growth and morphology, with activated Ras recruiting RalGDS to the membrane to activate Ral (*19*, *20*). The functional relationship of Ras and Ral is integral to understanding the mechanism of the interference of RalA function by ExoS, since the ADP-ribosylation of cellular Ras by ExoS has been shown to interfere with Ras activation and function (*12*−*14*). In examining whether ExoS could have a direct effect on RalA function, RalA binding to GST−RBD was found to be blocked by the ADP-ribosylation of RalA by ExoS in vitro, confirming the potential for ExoS to directly interfere with RalA binding to its downstream effector, RalBP1. The detection of minimal binding of cellular RalA to GST−RBD following bacterial translocation of ExoS, regardless of the state of Ral modification, also supports the fact that upstream effects of ExoS on Ras function might be affecting RalA activation. While the quality of the commercially available RalGDS antibody precluded definitive proof that ADP-ribosylation of Ras by ExoS affected RalGDS recruitment to the membrane, decreases in RalGDS membrane association were evident upon treatment of HT-29 cells with ExoS-producing bacteria (data not shown). We conclude from these studies that bacterially translocated ExoS affects RalA function, as evident by interference of RalA binding to its downstream effector, RalBP1, and that this interference can be mediated, at least in part, by the ADP-ribosylation of RalA by ExoS.

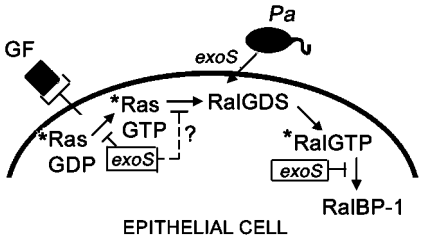

FIGURE 5: Effects of bacterially translocated ExoS on the Ras−Ral signal transduction pathway. Growth factor (GF) stimulation of eukaryotic cells results in activation of Ras. ExoS is translocated into eukaryotic cells by the type III secretory process of *P. aeruginosa* (*Pa*) where it efficiently ADP-ribosylates endogenous Ras and Ral (indicated by *****). ADP-ribosylation of Ras interferes with GEF-catalyzed Ras GDP to GTP exchange (*13*), which may affect Ras recruitment of RalGDS to the membrane. RalGDS, a GEF for Ral, activates Ral at the membrane, allowing Ral to interact with its downstream effector, Ral-BP-1. The ADP-ribosylation of Ral by ExoS interferes with the interaction of Ral−GTP with RalBP1.

A current understanding of the effects of ExoS ADPRT activity on cellular Ras-Ral signal transduction is summarized in Figure 5. The ADP-ribosylation of Ras by ExoS is known to interfere with Ras activation, which may affect the ability of Ras to interact and recruit its downstream effector, RalGDS, to the membrane, which leads to Ral activation. RalA is also efficiently ADP-ribosylated by bacterially translocated ExoS, and its ADP-ribosylation can sterically interfere with Ral binding to its downstream effector, RalBP1. Ral can mediate the activation of multiple cellular proteins, and while its precise function(s) within the cell is (are) not yet clearly understood, its vesicular localization, coupled with its binding to RalBP1 and Sec5, favors its role in endocytic and exocytic processes. Relating this to observed effects of ExoS on cell function, interference of Ral activation of filamin, RalBP1, and/or Sec5 by ExoS can explain the loss of cell surface structures, including filopodia, apparent in epithelial cells treated with ExoS-producing bacteria (*9*). Bacterially translocated ExoS is known to exert diverse and complex effects on epithelial cell function and has been found to ADP-ribosylate multiple LMWG-proteins within the cell including, in addition to Ras and RalA, members of the Rab and Rho families (*40*). The functional network of cellular signaling events affected by these LMWG-proteins complicates interpretations of the precise role of RalA modification in the observed effects of ExoS on eukaryotic cell function. While RalA appears to be an early target of bacterially translocated ExoS (*40*), more severe effects of ExoS are associated with cumulative substrate modification, suggesting that RalA represents one of many players in the complex effects of ExoS on eukaryotic cell function.

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