ADP Ribosylation of Arg41 of Rap by ExoS Inhibits the Ability of Rap To Interact with Its Guanine Nucleotide Exchange Factor, C3G†

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ABSTRACT: ExoS is a bifunctional type III cytotoxin that is secreted by *Pseudomonas aeruginosa*. The N-terminal domain comprises a RhoGAP activity, while the C-terminal domain comprises a ADP-ribosyltransferase activity. Previous studies showed that ExoS ADP ribosylated Ras at Arg41 which interfered with the ability of Ras to interact with its guanine nucleotide exchange factor. Rap and Ras share considerable primary amino acid homology, including Arg41. In this study, we report that ExoS ADP ribosylates Rap1b at Arg41 and that ADP ribosylation of Arg41 inhibits the ability of C3G to stimulate guanine nucleotide exchange. The mechanism responsible for this inhibition is one in which ADP-ribosylated Rap binds inefficiently to C3G, relative to wild type Rap. This identifies a second member of the Ras GTPase subfamily that can be ADP ribosylated by ExoS and indicates that ExoS can inhibit both Ras and Rap signaling pathways in eukaryotic cells.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that can cause serious infections in burn victims, cystic fibrosis patients, and the immunocompromised (*1*). *P. aeruginosa* is often refractory to treatment with antimicrobial agents, conferring concerns for infected individuals. *P. aeruginosa* utilizes several toxins to modify the physiology of eukaryotic cells (*2*). These include exotoxin A (PAETA) and type III cytotoxins, such as ExoS. While PAETA enters eukaryotic cells via receptor-mediated endocytosis, type III secreted toxins are translocated directly from bacteria into eukaryotic cells. Type III cytotoxins have been implicated in modulating the innate immune system of the host cell (*3*).

ExoS is a bifunctional type III cytotoxin. The N-terminus of ExoS is a GAP (GTPase-activating protein) for Rho GTPases (*4*), while the C-terminus ADP ribosylates eukaryotic proteins, including Ras (*5*). Expression of the ADP-ribosyltransferase domain is cytotoxic to cultured cells (*6*). ExoS ADP-ribosylates Ras at two sites, Arg41 and Arg128 (*7*). ADP ribosylation at Arg41 blocks the interaction of Ras with its guanine nucleotide exchange factor (GEF), Cdc25 (*8*). Guanine nucleotide exchange factors facilitate nucleotide exchange of monomeric G proteins. Inhibition of the Ras−Cdc25 interaction results in inactivation of the Ras signal transduction pathway since only GTP-bound Ras is capable of interacting with effector molecules such as Raf kinases.

The Ras subfamily of monomeric G proteins comprise a number of similar yet distinct proteins, including Ras, Rap, Ral, and TC21 (*9, 10*). Ras and Rap are more closely related than Ral and TC21, their sequences being >50% identical overall, and 100% identical in the switch 1 and switch 2 effector binding domains. Arginine 41 of Ras, the preferred site of ADP ribosylation by ExoS, is located at the C-terminal end of the switch 1 domain of Ras and is common to Ras and Rap (*11*). While similar at the primary amino acid level, Ras and Rap show unique interactions with upstream activators, GEFs, and downstream effectors, the Raf kinases.

The potential of ExoS to ADP ribosylate monomeric G proteins at their Arg41 homologues could be a tool for exploring the functional relationship between the switch I regions of Ras and Rap, while providing information about the capacity of ExoS to modulate host signal transduction pathways. This study shows that ExoS ADP ribosylates Rap at Arg41 and that ADP ribosylation at Arg41 inhibits the interaction of Rap with its guanine nucleotide exchange factor, C3G.

EXPERIMENTAL PROCEDURES

Materials. The following reagents were purchased: [^{32}P]-adenylate phosphate NAD and [^{32}P]αGTP (ICN), Sculptor in vitro mutagenesis kit and Glutathione Sepharose 4B (Amersham-Pharmacia), bovine serum albumin (BSA) and His-Probe (Pierce), DNA oligomers (Operon), and Ni^{2+}−agarose (Qiagen). All other chemicals were from Sigma unless otherwise noted. The plasmid encoding the catalytic domain of C3G (residues 830−1078) fused to GST is described elsewhere (*12*). The expression plasmid encoding FAS was obtained from H. Fu, and a plasmid encoding GST-Rap1b was obtained from K. Aktories. Hexahistidinyl ExoS

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containing the ADP-ribosyltransferase domain was purified as described previously (*13*).

Construction and Purification of His- and GST-Tagged Proteins. Expression vectors encoding N-terminal His(6) fusions of Rap1b or the catalytic domain of C3G (residues 830−1078) were constructed, using the following procedures. DNA encoding Rap1b was amplified by PCR and inserted into the pET30-Lic vector at the ligation independent sites, using methods and materials obtained from Novagen. DNA encoding C3G (residues 830−1078) was cleaved from pGEX-C3G at the *Bam*HI and *Sal*I restriction sites. A *Sal*I site was engineered into pET 15b (Novagen) downstream of the *Bam*HI site as a cassette (Operon), and DNA encoding C3G (residues 830−1078) was inserted into the *Bam*HI and *Sal*I sites of the modified pET 15b vector.

His(6) fusion proteins were purified as described previously (*13*). Briefly, *Escherichia coli* BL21(DE3) cells were suspended in Ni^{2+}−agarose binding buffer containing PMSF, aprotnin, leupeptin, RNase, and DNase. After a 2 h incubation and a subsequent 2 h induction [His(6)−Rap] or a 6 h incubation followed by overnight induction at 20 °C [His(6)−C3G(830−1078)] with 0.75 mM IPTG, cells were broken by passing them through a French press. Unbroken cells and insoluble material were removed by centrifugation at 20000*g* for 16 min. The His(6)−C3G(830−1078) fusion protein was purified from this soluble fraction by Ni^{2+}−agarose affinity chromatography. The purified His(6)−C3G(830−1078) fusion protein was diluted to 40% glycerol and stored as aliquots at −80 °C. The His(6)−Rap1b fusion protein was dialyzed overnight in 50 mM Tris-HCl (pH 7.6), containing 5 mM EDTA, 0.1% β-ME, and 10% glycerol, to remove bound nucleotides, before being frozen as aliquots at −80 °C. The GST−C3G(830−1078) fusion protein was purified as described previously (*12*) except that the lysed extract was batch purified on glutathione−Sepharose 4B resin and washed extensively. The GST−C3G(830−1078) fusion protein was analyzed in an immobilized state due to the insolubility of the released fusion protein.

Construction of the His(6)−Rap1b(R41Q/K) Fusion Protein. DNA encoding Rap1b was cloned into the *Xba*I and *Eco*RI restriction sites of M13-mp19. Site-directed mutagenesis was performed at codon Arg41, generating Gln and Lys mutations, using the Sculptor in vitro mutagenesis kit (Amersham). After mutagenesis, individual transfectants were sequenced and subcloned into the pET 30-Lic expression vector.

ADP Ribosylation of the His(6)−Rap1b Fusion Protein by ExoS. ADP ribosylation reaction mixtures contained (20 μL) 2.5 μM Rap1b, Rap1b(R41K), or Rap1b(R41Q), 0.1 mM [^{32}P]adenlyate phosphate NAD (specific activity of 0.125 Ci/mmol of NAD), 50 mM Tris-HCl, 0.25 μM FAS, and 2 μM BSA that were incubated with the catalytic deletion peptide, ExoS(233−453), at either 1.5 nM (linear velocity determinations) or 25 nM (stoichiometry determinations). Reactions were stopped by the addition of SDS−PAGE loading buffer and boiling. Samples were subjected to 13.5% SDS−PAGE, and gels were stained with Coomassie Blue. The band corresponding to the His(6)−Rap1b fusion protein was excised from the gel and counted in an LS 6000IC scintillation counter (Beckman).

Precipitations of Rap1b and ADPr-Rap1b with the GST−C3G and His(6)−C3G Fusion Proteins. Precipitation reactions were performed as follows: 5 μL of glutathione−Sepharose 4B (GST) resin (control) or 5 μL of resin with the immobilized GST−C3G(830−1078) fusion protein (200 pmol) was incubated with 2 mg/mL BSA for 30 min in C3G resuspension buffer [PBS with 10% glycerol, 0.1% β-ME, 5 mM EDTA, and 150 mM $(NH_4)_2SO_4$], washed, and suspended in 30 μL of 2 mg/mL BSA in C3G resuspension buffer. Resins were incubated with 50 pmol of nucleotide free Rap1b or ADP-ribosylated Rap1b at 4 °C for 30 min and centrifuged to pellet the resin. The resin was washed with 300 μL of PBS and suspended in 25 μL of SDS−PAGE loading buffer. The sample was subjected to SDS−PAGE, transferred to nitrocellulose, and probed by ECL (Super Signal, Pierce), using a His probe that was coupled to HRP following the manufacturer's instructions.

Association of Rap1b with the His(6)−C3G(830−1078) fusion protein was performed after removing the His(6) tag from Rap1b. The His(6) epitope was cleaved from 0.4 mg of the His(6)−Rap1b protein with thrombin [Pierce, 25 units/mg of the His(6)−Rap1b protein]. Thrombin-cleaved Rap1b was passed through a 1 mL column composed of 0.5 mL of benzamidine−Sepharose (Sigma) and 0.5 mL of Ni^{2+}−agarose buffer to remove thrombin and uncleaved His(6)−C3G protein, respectively. Fractions containing Rap1b were pooled in 10% glycerol prior to freezing aliquots at −80 °C. Rap1b or ADP-ribosylated Rap1b (200 pmol) was incubated with 300 pmol of the His(6)−C3G(830−1078) protein in 100 μL reaction mixtures for 30 min at 4 °C and then mixed with Ni^{2+}−agarose buffer (2.5 μL) for 30 min at 4 °C. Prior to mixing, the Ni^{2+}−agarose buffer was incubated with 1 mg/mL BSA for 30 min at 4 °C to reduce the level of nonspecific binding to the resin. The reaction mixture was centrifuged, and the agarose was washed and suspended in SDS−PAGE loading buffer. Samples were subjected to SDS−PAGE, transferred to nitrocellulose, and probed by ECL, using monoclonal anti-Rap1 (Signal Transduction Laboratories, 1/500) as the primary antibody. Ni^{2+}−agarose precipitations without the His(6)−C3G(830−1078) protein did not bind detectable amounts of Rap1b (data not shown).

Guanine Nucleotide Exchange Assays with Rap1b and the His(6)−C3G Protein. Nucleotide loading of GTPase was performed as follows (25 μL reaction mixtures): 0.4 μM nucleotide-free Rap1b or ADPr-Rap1b was incubated with 10 μM [^{32}P]αGTP (specific activity of 8 Ci/mmol of GTP) in 40 mM Tris-HCl (pH 7.6) and 2 mM EDTA for 10 min at 4 °C (level of nucleotide binding of >30%). At this time, intrinsic nucleotide exchange or guanine nucleotide exchange factor-stimulated exchange, in the presence of 6.6 μM His(6)−C3G(830−1078), was initiated by the addition of high-salt exchange buffer [2 mM DTT, 1 mM GTP, 20 mM $MgCl_2$, 500 mM NaCl, and 40 mM Tris-HCl (pH 7.6)]. At the indicated times, duplicate samples (55 μL) were spotted onto nitrocellulose filters (Millipore) and filters were washed with 10 mL of wash buffer [40 mM Tris-HCl (pH 7.6), 0.1% β-ME, and 10 mM $MgCl_2$] at 4 °C. The amount of radioactivity was measured in an LS 6000IC scintillation counter (Beckman). The percentage of GTP bound to Rap1b was calculated relative to the amount of GTP bound to Rap1b at time zero. Each experiment was performed in duplicate and repeated at least once.

Table 1: ADP Ribosylation of Rap1b by ExoS[a]

protein	stoichiometry of ADP ribosylation (mol of NAD/ mol of Rap)	linear velocity of ADP ribosylation [mol of NAD min^{-1} (mol of ExoS)$^{-1}$]
wild type Rap1b	1.5 ± 0.06	62 ± 9
Rap1b(R41K)	0.9 ± 0.04	29 ± 4

[a] The stoichiometry was determined from 20 μL reaction mixtures containing 2.5 μM recombinant His(6)−Rap1b, 25 nM ExoS(233−453), 2 μg of BSA, 0.1 mM [^{32}P]adenylate phosphate NAD, and 0.25 μM FAS in 50 mM Tris-HCl (pH 7.6). The reaction mixture was incubated at room temperature for 60 min and the reaction stopped by the addition of SDS−PAGE loading buffer. The samples were run on a gel and stained with Coomassie Blue. Protein bands were excised, and the amount of radioactivity was measured in a scintillation counter. The linear velocity was measured as described in the legend of Figure 1. Values are averages of three independent trials.

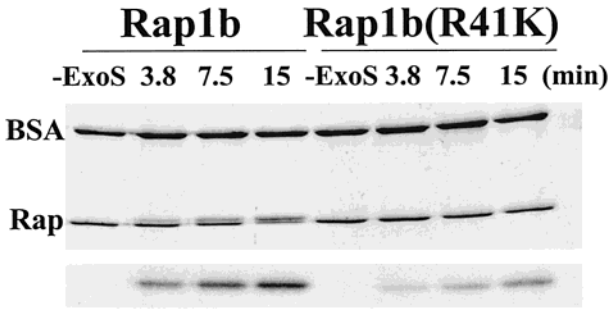

FIGURE 1: Exoenzyme S ADP ribosylates Rap1b at Arg41 in vitro. (Top) Coomasie Blue-stained SDS−PAGE gel of in vitro linear velocity ADP ribosylation reactions. The recombinant His(6)−Rap1b or His(6)−Rap1b(R41K) fusion protein (2.5 μM) was incubated with 2 μg of BSA (−ExoS) or 1.5 nM ExoS and 2 μg of BSA in a 20 μL reaction mixture containing 0.1 mM [^{32}P]adenylate phosphate NAD (specific activity of 0.125 Ci/mmol of NAD), 50 mM Tris-HCl (pH 7.6), and 0.25 μM FAS. Reactions were stopped at 3.8, 7.5, or 15 min with SDS−PAGE loading buffer. Samples were analyzed by 13.5% SDS−PAGE; the bands of migration of BSA and Rap1b are indicated. (Bottom) The autoradiogram of this gel shows the incorporation of radiolabel into the His(6)−Rap and His(6)−Rap1b(R41K) proteins.

RESULTS

Exoenzyme S ADP Ribosylates Rap1b at Arginine 41. Earlier studies determined that ExoS ADP ribosylated Ras at Arg41 and Arg 128, with Arg41 being the preferred site of ADP ribosylation, and that ADP ribosylation of Arg41 interfered with Ras binding its guanine nucleotide exchange factor, Cdc25 (*8*). Although the sequences of Rap and Ras are considerably homologous, it was not apparent if Rap would be ADP ribosylated by ExoS or if ADP ribosylation would modulate Rap function. Initial experiments showed that ExoS ADP ribosylated Rap1b and Ras with similar linear velocities (data not shown). At high concentrations of ExoS, ~1.5 mol of ADP ribose was incorporated per mole of wild type Rap1b, while 0.9 mol of ADP ribose was incorporated per mole of either Rap1b(R41Q) or Rap1b(R41K) (Table 1). Linear velocity analysis showed that ExoS ADP ribosylated wild type Rap1b at approximately twice the rate of Rap1b(R41Q) or Rap1b(R41K) (Figure 1 and Table 1). These data indicated that ExoS ADP ribosylated Rap at two sites, Arg41 and a second undetermined site, and that both sites were equally accessible to ADP ribosylation. ADP ribosylation of Ras at Arg41 shifted the electrophoretic mobility of Ras upon SDS−PAGE (*14*). ADP ribosylation of Rap1b also produced a shift in electrophoretic mobility (Figure 1, top), while the electrophoretic shift was less apparent for ADP-ribosylated Rap(R41K) (Figure 1, top). Extended electrophoresis allowed resolution of the shift in the mobility of ADP-ribosylated Rap(R41K) (Figure 2B).

ADP Ribosylation of Rap1b at Arg41 Blocks Rap1b−C3G Interaction. The effect of ADP ribosylation at Arg41 on the ability of Rap to interact with its guanine nucleotide exchange factor, C3G, was determined. To date, direct binding of C3G and Rap has not been reported. The catalytic domain of C3G(830−1078) was expressed as a GST fusion protein and immobilized on glutathione gel. The immobilized GST−C3G(830−1078) fusion protein bound Rap1b more efficiently than ADP-ribosylated Rap1b (Figure 2A). Under the experimental conditions that were employed, glutathione−Sepharose did not precipitate either Rap1b or ADP-ribosylated Rap1b (Figure 2A). When equal amounts of Rap1b and ADP-ribosylated Rap1b were incubated together with the immobilized GST−C3G protein, only Rap1b was efficiently precipitated (Figure 2A). These data indicated that C3G−Rap1b interactions could be assessed in a precipitation reaction and that ADP ribosylation reduced the affinity of Rap1b for its guanine nucleotide exchange factor, C3G.

Next, experiments were performed to determine if ADP ribosylation of Rap1b at Arg41 was responsible for the observed inhibition of Rap1b−C3G interactions. The immobilized GST−C3G(830−1078) protein was incubated with Rap(R41K) and ADP-ribosylated Rap1b(R41K) and precipitated. In contrast to wild type Rap1b, comparable amounts of Rap1b(R41K) and ADP-ribosylated Rap1b(R41K) were precipitated with the immobilized GST−C3G(830−1078) protein, using $^1/_2$ the amount of each protein in the precipitation reaction (Figure 2B). Although the differential migration elicited by ADP ribosylation of Rap1b(R41K) was less apparent than that of wild type Rap1b, extended electrophoresis allowed resolution of ADP-ribosylated Rap1b(R41K) and Rap1b(R41K). When equal amounts of Rap1b(R41K) and ADP-ribosylated Rap1b(R41K) were incubated together with the immobilized GST−C3G protein, both forms were efficiently precipitated (Figure 2B). Together, these data indicated that ADP ribosylation of Rap1b at Arg41 physically interfered with the Rap1b−C3G interaction. In addition, these studies showed that replacement of Arg41 with Lys did not abrogate the binding of Rap1b to C3G.

To corroborate the ability of ADP ribosylation to interfere with the ability of C3G to interact with Rap1b, experiments were designed to determine the effect of ADP ribosylation on the C3G-stimulated nucleotide exchange of Rap1b. However, under our assay conditions, the GST−C3G(830−1078) protein stimulated incomplete nucleotide exchange (*12*). This limited measurement of the effect of ADP ribosylation on the exchange capacity of C3G prompted the engineering of a new form of C3G, His(6)−C3G(830−1078), which efficiently stimulated nucleotide exchange in Rap1b.

The His(6)−C3G Protein Is an Active Nucleotide Exchange Factor for Rap1b. The recombinant His(6)−C3G protein was expressed at approximately 25 mg of His(6)−C3G(830−1078)/L of culture in *E. coli* BL21(DE3) cells and was efficiently purified by Ni^{2+} affinity chromatography (Figure 3, inset). This exceeded the level of expression of the GST−C3G(830−1078) protein, which was reported to

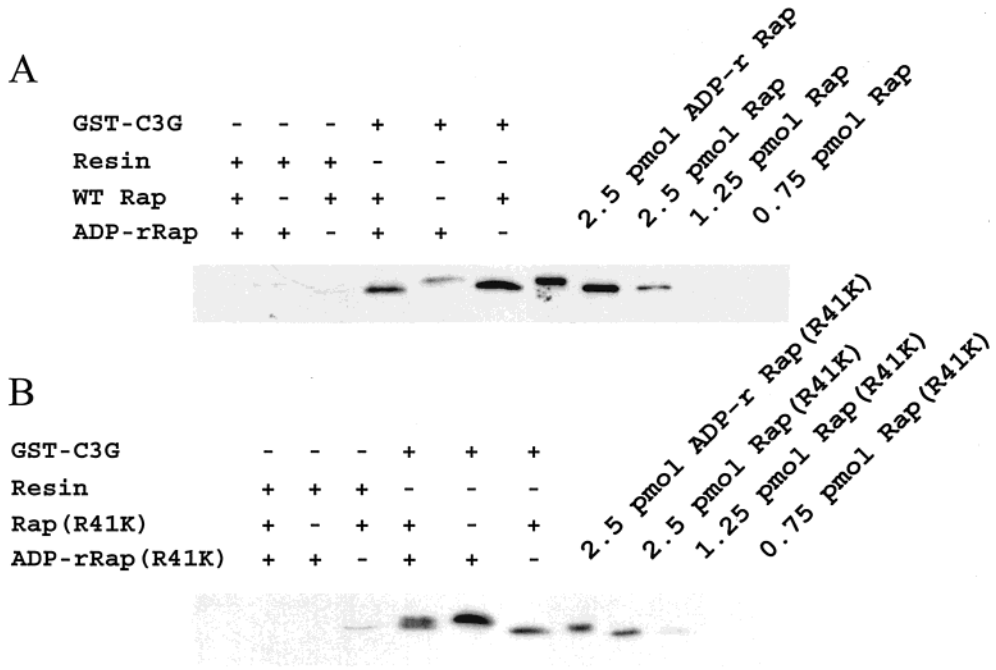

FIGURE 2: ADP ribosylation of Rap at Arg41 blocks interaction of Rap with the GST−C3G protein. (A) Rap1b and ADPr-Rap1b (2.5 μM) or Rap (1.25 μM) and ADPr-Rap (mixing experiment) were added to 200 pmol of GST−C3G(870−1037) and glutathione−Sepharose (5 μL of resin) or glutathione−Sepharose (5 μL of resin) in the presence of 2 mg/mL BSA for 30 min at 4 °C. The reaction mixtures were pelleted, washed, and suspended in SDS−PAGE sample buffer. Each mixture was subjected to 13.5% SDS−PAGE, and proteins were transferred to a nitrocellulose filter. The presence of the His(6)−Rap protein was determined by subjecting the filter to ECL, using the His−probe−HRP conjugate as the reactive probe (Pierce). (B) Reactions identical to those described for panel A were performed with the His(6)−Rap(R41K) protein.

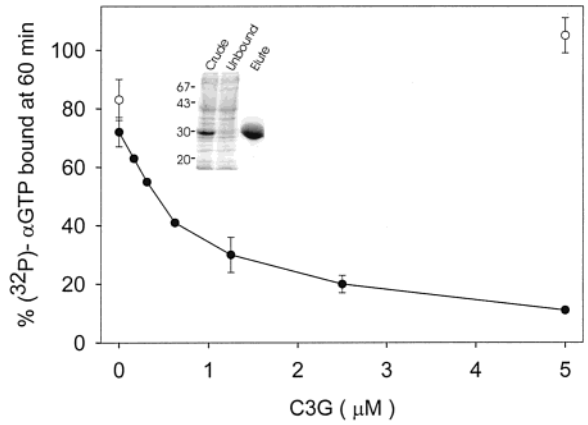

FIGURE 3: His(6)−C3G is an effective exchange factor for Rap1b. Rap1b [400 nM (●)] or Ha-Ras [400 nM (○)] was loaded with [γ-^{32}P]GTP and incubated with the indicated amount of the His(6)−C3G protein. After 60 min at room temperature, the amount of bound radiolabel was determined as nitrocellulose binding material. Values are the averages of at least two experiments. (Inset) Purification of the His(6)−C3G protein. The crude lysate of *E. coli* BL21(DE3) cells containing the pET15b−C3G(830−1078) protein (crude) was subjected to Ni^{2+}−agarose chromatography; the unbound material (unbound) and imidazole-eluted material (elute) were subjected to 13.5% SDS−PAGE, and the gel was stained with Coomassie Blue. The major Coomassie staining band in the elute fraction corresponds to C3G(830−1078), which was used in this study.

be produced at 0.5 mg/L of culture (*12*). During the initial characterization of the His(6)−C3G(830−1078) protein, it was apparent that this fusion protein possessed limited solubility in buffers commonly used to assess nucleotide exchange, such as 40 mM Tris-HCl (pH 7.6), 2 mM DTT, and 20 mM $MgCl_2$ (data not shown). An empirical evaluation of buffer conditions, however, showed that the His(6)−C3G-(830−1078) protein was soluble in buffers containing 0.5 M NaCl. Subsequent experiments were performed in high-ionic strength buffers where the His(6)−C3G(830−1078) protein was found to stimulate complete nucleotide exchange in Rap1b (Figure 3). The specific activity of the His(6)−C3G(830−1078) protein for stimulation of Rap1b nucleotide exchange was similar to the reported initial velocity of the GST−C3G(830−1078) protein. Our inability to detect GST−C3G(830−1078) stimulation of Rap nucleotide exchange could have been due to the ionic strength of the buffers used in the earlier studies, which limited the resolution of our assay.

ADP Ribosylation of Rap1b at Arg41 Inhibits C3G-Mediated Guanine Nucleotide Exchange. The effect of ADP ribosylation on His(6)−C3G(830−1078)-stimulated nucleotide exchange of Rap1b was examined. Although the intrinsic rate of nucleotide exchange was similar for both Rap1b and ADP-ribosylated-Rap1b (Figure 4A), the rate of His(6)−C3G(830−1078)-stimulated nucleotide exchange was enhanced approximately 3-fold for Rap1b versus ADP-ribosylated Rap1b when measured at the 60 min time point (Figure 4A). Extended incubation of the His(6)−C3G protein and either Rap1b or ADP-ribosylated Rap1b resulted in >90% nucleotide exchange (data not shown). These data indicated that ADP ribosylation interfered with C3G-mediated guanine nucleotide exchange of Rap1b.

To determine if the decrease in the level of C3G-mediated nucleotide exchange of ADP-ribosylated Rap1b was due to ADP ribosylation at Arg41, His(6)−C3G(830−1078)-stimulated nucleotide exchange was performed on Rap-(R41K) and ADP-ribosylated Rap(R41K). Figure 4B shows

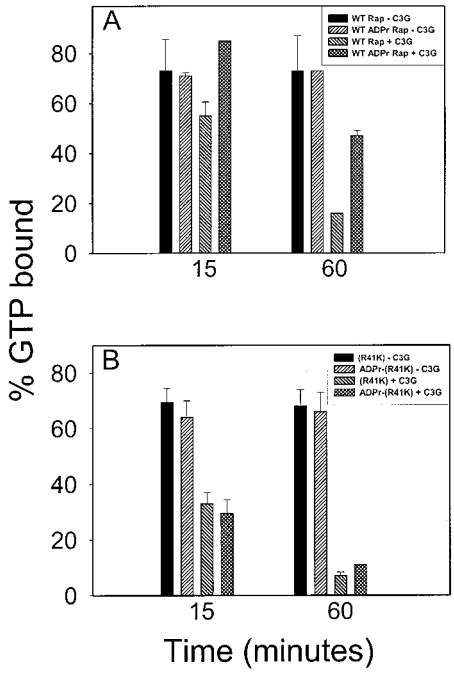

FIGURE 4: ADP ribosylation of Rap at Arg41 reduces the level of C3G-mediated guanine nucleotide exchange of Rap. (A) The unmodified and ADP-ribosylated His(6)−Rap1b protein were assessed for their ability to release bound [^{32}P]αGTP in the presence and absence of the His(6)−C3G protein. Rap (0.4 μM) was loaded with radiolabeled [^{32}P]αGTP, and 6.6 μM His(6)−C3G was added where indicated. Nucleotide exchange was started by the addition of 10 mM $MgCl_2$ and a 100-fold excess GTP. Reaction mixtures were filtered through nitrocellulose followed by washing. The amount of filter-bound radiolabel was determined by scintillation counting. Values are the averages of two representative experiments. Panel B is identical to panel A except that the His(6)−Rap1b(R41K) protein is used in the reaction mixtures instead of the His(6)−Rap1b protein.

that C3G-mediated guanine nucleotide exchange was identical for both Rap(R41K) and ADP-ribosylated Rap(R41K). Together, these data indicate that the ADP ribosylation of Rap1b at Arg41 interferes with the ability of Rap1b to interact with its guanine nucleotide exchange factor. In addition, the data indicated that Arg41 does not contribute to Rap1b binding to C3G.

ADP Ribosylation of Rap1b Blocks Interaction of Rap with the His(6)−C3G(830−1078) Protein. Next, the interaction of Rap1b and ADP-ribosylated Rap1b with the His(6)−C3G-(830−1078) protein was assessed. Ni^{2+}−agarose buffer was used to precipitate the His(6)−C3G(830−1078) protein from a reaction mixture containing the His(6)−C3G(830−1078) protein and either Rap1b or ADP-ribosylated Rap1b. The amount of Rap1b or ADP-ribosylated Rap1b present in the His(6)−C3G precipitate was determined by Western blot analysis, probing with the Rap1b monoclonal antibody. While Rap1b was precipitated with the His(6)−C3G(830−1078) protein, ADP-ribosylated Rap1b was not precipitated with the His(6)−C3G(830−1078) protein (Figure 5). This indicated that ADP-ribosylated Rap1b had a lower affinity for the His(6)−C3G protein than Rap1b. Control experiments showed that this monoclonal anti-Rap antibody recognized Rap1b(R41K) weakly, which prevented an analysis of the interaction of the His(6)−C3G(830−1078) protein with Rap1b(R41K).

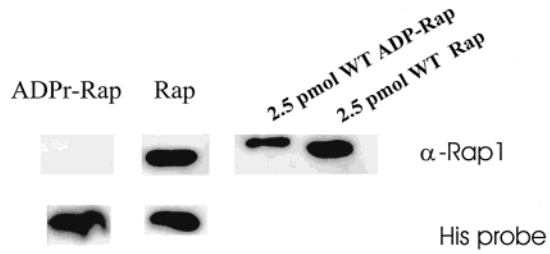

FIGURE 5: ADP ribosylation of wild type Rap blocks interaction of Rap with the His(6)−C3G protein. Rap1b or ADP-ribosylated Rap1b (1.8 μM) was incubated in the presence of 4 μM His(6)−C3G(830−1078) and precipitated with Ni^{2+}−agarose buffer. Precipitates were suspended in 20 μL of sample buffer and subjected to 13.5% SDS−PAGE. The gel was transferred to nitrocellulose, and the nitrocellulose was subjected to ECL using either the anti-Rap1b antibody or the His probe as the primary antibody or reagent, respectively. The experiment was repeated twice, and a representative blot is shown.

DISCUSSION

ExoS was identified as the second ADP-ribosyltransferase produced by *P. aeruginosa*, the first being exotoxin A (*15*). Coburn and Gill identified two unique properties of ExoS. First, ExoS required a eukaryotic protein, termed FAS, for expression of ADP-ribosyltransferase activity (*16*). The activator was later shown to be a 14-3-3 protein (*17*). Second, ExoS ADP ribosylated several proteins within a eukaryotic cell lysate, with Ras being a preferred target for ADP ribosylation (*5*). Subsequent studies showed that ExoS ADP ribosylated Ras at two arginines, Arg41 and Arg128 (*7*). Although the physiological significance of the ADP ribosylation of Arg128 was not resolved, ADP ribosylation of Ras at Arg41 uncoupled Ras signal transduction, interfering with the binding of the guanine nucleotide exchange factor to Ras (*8*). Transient transfections showed that the expression of the ADP-ribosyltransferase domain of ExoS was cytotoxic to eukaryotic cells and that this cytotoxicity was not reversed by expression of a dominant active form of Ras (M. J. Riese and J. T. Barbieri, unpublished data). Together, these data indicated that inhibition of Ras signaling was not responsible for the cytotoxic phenotype elicited by ExoS. The current study was initiated to determine if ExoS ADP ribosylated other Ras GTPases. Our studies showed that ExoS ADP ribosylated Rap1b at two sites, including Arg41, and that ADP ribosylation of Rap1b(R41Q) inhibited the ability of C3G to stimulate guanine nucleotide exchange. The mechanism responsible for this inhibition was via steric hindrance, since C3G bound Rap better than ADP-ribosylated Rap. ADP ribosylation of Rap did not alter intrinsic nucleotide exchange, which indicated that ADP ribosylation at Arg41 did not introduce functional changes into Rap. Although our understanding of how Rap modulates eukaryotic signal transduction is limited, current studies predict that the inhibition of Rap activation by ADP ribosylation could modulate several Rap-dependent phenotypes, including activation of oxidative burst in neutrophils (*18*) or transduction through integrin-mediated signaling pathways (*19*). While in vivo ADP ribosylation of Ras and Ral have been observed, earlier attempts to assess the in vivo ADP ribosylation of Rap by ExoS were unsuccessful (*20*). This could be due a combination of events related to either the modest shift in the migration of Rap (relative to Ras) upon ADP ribosylation, the quality of antisera available to probe

Rap expression, or the amount of Rap ADP ribosylated in vivo. Our current studies include the generation of a eukaryotic vector that will express sufficient Rap to allow characterization of this GTPase in cultured cells.

Rap was originally identified in a screen as an antagonist of Ras-mediated transformation (*21*). While Ras-GTP and Rap-GTP interact similarly with Raf1 kinase at the Raf binding domain (residues 51−131) (*22*), Ras-GTP and Rap-GTP interact differently with Raf1 kinase in the cysteine-rich region (residues 132−206) (*23*). The differential ability of Rap and Ras to activate Raf kinase may produce the opposing cellular signaling effects. Rap binds Raf1, but fails to activate Raf1 kinase activity, while Ras binds and activates Raf1. Thus, in cells in which Ras signals through Raf1, Rap could inhibit Ras signaling. B-Raf is a Raf isoform that is present in brain and testes and is activated by both Ras and Rap. Although controversial, high-level expression of B-Raf could augment the function of both Ras and Rap (*24*). Ras and Rap are the most closely related members of the Ras subfamily of GTPases, with essentially identical switch I and switch II domains. Despite this homology, mutations within the switch I region may yield similar or different phenotypes with respect to interactions with their respective guanine nucleotide exchange factors, GEFs (*25*). For example, the S17N mutation in Ras confers a dominant negative phenotype in vivo, while the S17N mutation in Rap confers a null phenotype. Mechanistic studies showed that Ras(S17N) binds tightly to GEFs, preventing GEF-mediated activation of other Ras molecules. In contrast, Rap(S17N) does not show this dominant negative phenotype on its GEF, C3G. GEF interactions with Rap and Ras also differ within their switch 1 regions. The Y40C mutation in Rap does not interfere with C3G-mediated nucleotide exchange, while the Y40C mutation within Ras reduces the level of Cdc25-mediated nucleotide exchange. Because Rap signaling was intertwined in Ras signal transduction, it was difficult to predict the cellular effects of ADP ribosylation on Rap1b. Our studies showed that ADP ribosylation of Ras and Rap yields essentially identical functional phenotypes and resulted in an inhibition of the binding of the exchange factor to their respective GTPase. This indicates that the C-terminal regions of the switch I domains of Ras and Rap share functional and topological properties. Thus, the observed differences of the Y40C mutation in Ras and Rap may be due to interaction with regions of the respective GTPase distant from the switch 1 region rather than to interactions with regions contiguous to the switch 1 region. However, steric considerations must be employed when interpreting the ability of ADP ribosylation to inhibit protein−protein interactions.

A number of bacterial toxins modify G proteins in switch 1 and switch 2 regions. A paradigm has been established in which modification of the switch 1 region leads to inactivation of the respective G protein and modification of the switch 2 region leads to constitutive activation of the G protein. For example, ADP ribosylation of Asn41 of Rho by the C3 toxin from *Clostridium botulinum* inhibits the ability of the GTPase to stimulate Rho-dependent functions (*26*), while cytotoxic necrotizing factor 1 from *E. coli* deamidates Gln63 of Rho, which inhibits GTPase activity (*27*). ExoS follows that model, since ADP ribosylation of Ras or Rap1b within its switch I region (Arg41) inhibits interactions of the GTPase with its respective exchange factor.

Completion of this study required the engineering of a form of C3G that retained exchange factor capabilities. Previous bacterial expression of the exchange factor domain of C3G(830−1078) utilized a GST fusion strategy (*25*). This recombinant protein was instrumental in allowing the analysis of mutated forms of Rap, which defined the function of numerous residues. The His(6)−C3G(830−1078) protein also appears to be a useful form of the exchange factor, being well expressed in bacteria. The ability to produce soluble His(6)−C3G(830−1078) protein should extend studies on the interaction between Rap and C3G by allowing the direct physical assessment of interactions between Rap and its exchange factor.

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