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ADP-Ribosylation of p21^{ras} and Related Proteins by *Pseudomonas aeruginosa* Exoenzyme S

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***Pseudomonas aeruginosa* exoenzyme S ADP-ribosylates p21^{ras} and several related proteins. ADP-ribosylation of p21^{ras} does not alter interactions with guanine nucleotides. The *ras*-related GTP-binding proteins, including Rab3, Rab4, Ral, Rap1A, and Rap2, are also substrates; given these results, we propose a model for the role of exoenzyme S in pathogenesis.**

Pseudomonas aeruginosa exoenzyme S preferentially ADP-ribosylates p21$^{c\text{-}Hras}$ and several other 21- to 25-kDa GTP-binding proteins (12). Other bacterial ADP-ribosyltransferases also modify GTP-binding proteins, with resulting functional changes (5, 6, 9, 25, 31). Of these ADP-ribosyltransferases, it is cholera toxin with which exoenzyme S shares the most characteristics. Exoenzyme S and cholera toxin fragment A1 both ADP-ribosylate a number of proteins in vitro in addition to the preferred G proteins (10, 21), both modify arginine residues (12, 27), and each requires a specific eukaryotic protein for full enzymatic activity (11, 21–23). ADP-ribosylation by cholera toxin decreases the GTPase activity of Gsα (5), and this effect has been directly related to the pathogenesis of *Vibrio cholerae* infection. Exoenzyme S has not been definitively proven to be a toxin, but it does appear to contribute to pathogenesis (28, 29). To understand how ADP-ribosylation catalyzed by exoenzyme S might contribute to the pathogenesis of *P. aeruginosa* infections, we have identified low-molecular-weight GTP-binding proteins, other than p21^{ras}, which are substrates for exoenzyme S and have looked for effects of ADP-ribosylation on p21^{ras} interactions with guanine nucleotides.

To determine whether various p21^{ras}-related proteins were ADP-ribosylated by exoenzyme S, we tested several purified *ras*-related gene products which were expressed in *Escherichia coli*. Figure 1 shows that the products of the c-H-*ras*, *rap1A*, *rap2*, *ral*, *rab3*, and *rab4* genes (7, 11, 34, 37) were [^{32}P]ADP-ribosylated by exoenzyme S, while Rab1 and RhoC (26), ARF (36), and Gp (15) were not. ADP-ribosylation of each protein was dependent on the addition of FAS, a eukaryotic protein factor required for exoenzyme S enzymatic activity (11). The conditions used (see the legend to Fig. 1) allowed only partial ADP-ribosylation of substrates (12) and were chosen to determine the relative abilities of these proteins to be modified by exoenzyme S. ARF, RhoC, Gp, and Rab1 were not ADP-ribosylated, even with 10-fold-higher exoenzyme S concentrations.

The ability to bind and hydrolyze GTP is central to the normal functions of all GTP-binding proteins (3). We therefore set out to determine the effects of exoenzyme S-catalyzed ADP-ribosylation on p21^{ras} interactions with guanine nucleotides in vitro. GTP binding by ADP-ribosylated and unmodified p21^{ras} was measured essentially as described previously (13). For these studies, we used A2.1-1 cells, derivatives of NIH 3T3 cells that overexpress the c-H-*ras* gene (24). A2.1-1 cells were metabolically labeled with [^{35}S]methionine, washed, and harvested by scraping. The cells were lysed by freezing and thawing, and the membranes were washed and collected by centrifugation. A2.1-1 cell membranes were ADP-ribosylated by exoenzyme S in the presence of unlabeled NAD. Controls contained buffer in place of exoenzyme S. p21^{ras} was immunoprecipitated with rat monoclonal antibodies YA6-172 and Y13-259 (20). Nor-

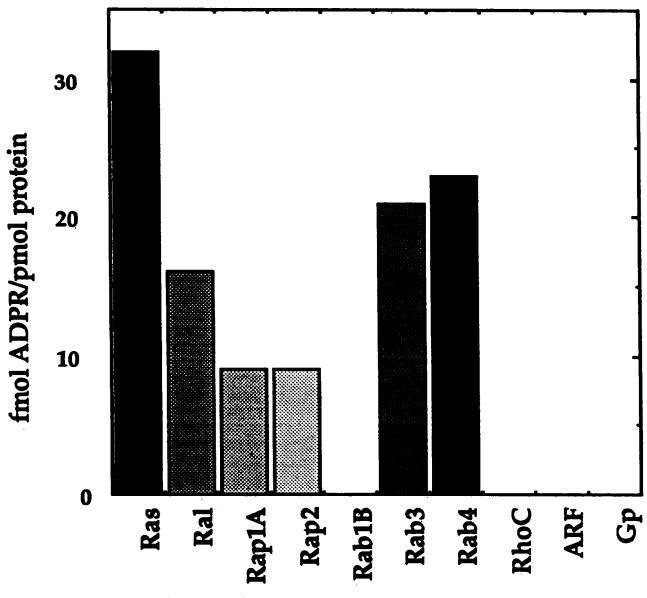

FIG. 1. ADP-ribosylation of p21^{ras} and related proteins by exoenzyme S. The purified proteins (0.5 to 1 μg each) were incubated with 10 μg of FAS per ml, 0.3 μg of exoenzyme S per ml, and 5 μM [^{32}P]NAD in a total volume of 15 μl of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–130 mM NaCl (pH 7.3) for 30 min at 25°C. After fractionation on 12.5% polyacrylamide gels under denaturing conditions, the [^{32}P]ADPR-protein bands were excised and ADPR incorporation was quantitated by liquid scintillation counting. The extent of ADP-ribosylation (femtomoles) was compared with the amount of each protein (picomoles); the ratio is shown. ADPR, ADP-ribose.

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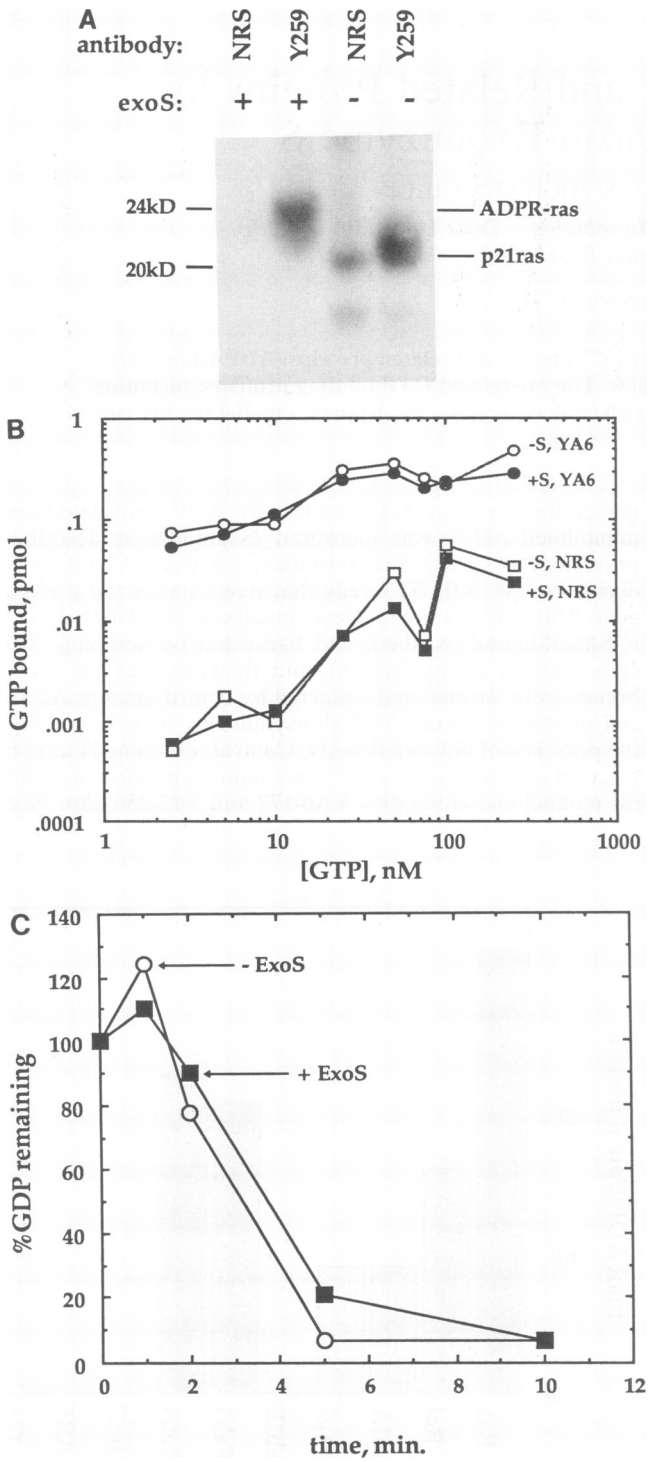

FIG. 2. Nucleotide binding and exchange by p21$^{c\text{-}H\text{-}ras}$ and ADPR-p21$^{c\text{-}H\text{-}ras}$. A2.1-1 cells were metabolically labeled with [^{35}S]methionine and ADP-ribosylated in the presence of 3 μg of exoenzyme S per ml (closed symbols) or buffer (open symbols) and 10 mM unlabeled NAD in HEPES-NaCl (see legend to Fig. 1). After 1 h at 25°C, p21^{ras} was immunoprecipitated by rat monoclonal antibodies YA6-172 and Y13-259 (see Fig. 3) and protein A-Sepharose which had been coated with anti-rat immunoglobulin G developed in goats. Normal rat serum (NRS) was used as a control. A portion of each sample was fractionated on a 12.5% polyacrylamide gel to determine the extent of ADP-ribosylation, and the gel was exposed to X-ray film (A). Results for Y13-259 are shown; similar results were obtained with YA6-172. Positions of marker proteins are shown. The remaining portions of the samples were divided for two experiments. For the binding experiment shown in panel B, the beads were incubated with various amounts of [α-^{32}P]GTP (NEN) in 50 μl of 20 mM Tris-HCl (pH 8.0)–125 mM NaCl–2 mM $Mg(C_2H_3O_2)_2$–1 mM $CaCl_2$–1 mM dithiothreitol–1% Triton X-100 (immunoprecipitation buffer [IPB]) for 1 h at 32°C. The beads were collected on glass fiber filters, and the excess unbound GTP was removed by washing with IPB. Bound GTP was quantitated in a liquid scintillation counter. For the exchange experiment shown in panel C, the beads were incubated for 1 h at 4°C with 10 nM [α-^{32}P]GDP (16), washed, and diluted into 100 volumes of IPB containing 500 μM unlabeled GTP. Samples were removed at the times indicated and washed under suction on glass fiber filters. The filters were counted in a liquid scintillation counter. These data are representative of three experiments.

mal rat serum was used as a control for immunoprecipitation. After immunoprecipitation, a portion of each sample was fractionated on a polyacrylamide gel under denaturing conditions to ensure that ADP-ribosylation was complete (Fig. 2A). The remainder of each sample was incubated for 1 h with various concentrations of [α-^{32}P]GTP and washed on glass fiber filters to remove excess [^{32}P]GTP. Bound GTP was quantitated by scintillation counting. ADP-ribosylation had no significant effect on the ability of p21^{ras} to bind GTP (Fig. 2B). In a similar experiment, GTP-for-GDP exchange (measured as described previously [16]) by p21^{ras} was also not affected by ADP-ribosylation (Fig. 2C). The exchange rate shown here is consistent with previously published results (17). In addition, the interaction of p21^{ras} with GRF, the protein which accelerates the release of GDP from *ras* proteins (35), was not altered by ADP-ribosylation (data not shown).

The GTPase activities of ADP-ribosylated p21^{ras} and of unmodified p21^{ras} were measured in the presence and absence of purified GAP (GTPase activator protein [1]). The method was modified from that of Cales et al. (4). Pigeon erythrocyte membranes (36) and A2.1-1 cell membranes were used for this experiment. The membranes were incubated for 1 h at 25°C with unlabeled NAD and exoenzyme S or with the exoenzyme S buffer. A portion of each A2.1-1 cell sample was immunoprecipitated as described above to determine the extent of ADP-ribosylation. [α-^{32}P]GTP was allowed to bind to the remaining portions of the samples as described previously (4), and the samples were incubated with either 36 μg of GAP per ml or the buffer control. Samples were removed at the times indicated in Fig. 3 and immunoprecipitated with p21^{ras}-specific monoclonal antibody Y13-259 (20, 33). p21^{ras}-associated nucleotides were fractionated by thin-layer chromatography. ADP-ribosylation altered neither the intrinsic nor the GAP-stimulated GTPase of p21^{ras} (Fig. 3).

Bacterial ADP-ribosyltransferases have been shown to disrupt GTP-binding protein function in a variety of ways. Cholera toxin-catalyzed ADP-ribosylation decreases the GTPase activity of Gsα, but several other toxins affect G protein function without changing interactions with GTP (9, 25, 30, 32). Pertussis toxin-catalyzed ADP-ribosylation alters the interaction of Gi with the receptor (25), while ADP-ribosylation by exotoxin A or diphtheria toxin affects the functional interaction of EF-2 with the ribosome (9). Our results show that ADP-ribosylated p21^{ras} is still able to bind, exchange, and hydrolyze guanine nucleotides normally. However, the larger-than-expected change in the electro-

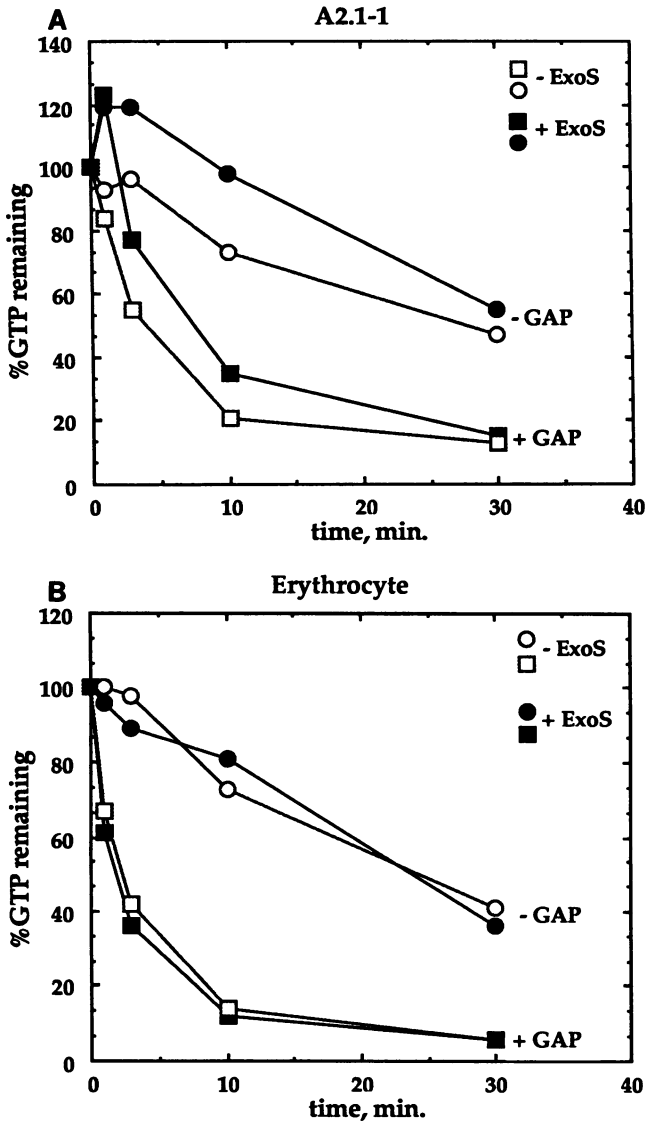

FIG. 3. Intrinsic and GAP-stimulated GTPases of native and ADP-ribosylated p21^{ras}. A2.1-1 cell membranes (A) and pigeon erythrocyte membranes (B) were incubated with unlabeled NAD and exoenzyme S (closed symbols) or buffer for the controls (open symbols) as described for Fig. 2. A portion of each A2.1-1 cell sample was immunoprecipitated with goat anti-rat immunoglobulin G-coated protein A-Sepharose beads and p21^{ras}-specific monoclonal antibody Y13-259 or normal rat serum as a control and fractionated on a 12.5% polyacrylamide gel (as in Fig. 2A). The membranes were washed and resuspended in HEPES-NaCl (see legend to Fig. 1) plus 1 mM dithiothreitol, 10 mM EDTA, 5 mM $Mg(C_2H_3O_2)_2$, 100 μM ATP, 20 μM ammonium vanadate, and 1.25 μM [α-^{32}P]GTP (NEN). After 5 min at 30°C, the magnesium concentration was raised to 30 mM and GAP (36 μg/ml) (squares) or the buffer control (circles) was added. All samples were immunoprecipitated as described above. The beads were boiled for 3 min in 1 volume of 0.1% sodium dodecyl sulfate–1 mM EDTA. Samples (1 μl) were fractionated on polyethyleneimine-cellulose thin-layer plates developed in 0.75 M potassium phosphate (pH 3.4). The plates were cut, and the pieces were counted in a liquid scintillation counter to quantitate GTP and GDP. These data are representative of three experiments.

phoretic mobility of p21^{ras} upon ADP-ribosylation (Fig. 2A) (12) suggests that the modification causes a conformational change in the p21^{ras} protein which may have an impact on its function. Similar large shifts in electrophoretic mobility upon ADP-ribosylation were seen with Rap1A, Rap2, and Ral. Our results suggest that any alterations in the functions of p21^{ras} and the other related substrates may be manifested through interactions with other cellular proteins rather than through changes in interactions with guanine nucleotides. Low-molecular-weight GTP-binding proteins related to p21^{ras}, especially the *rab* gene products, have been identified as components of endocytic and exocytic vesicle trafficking pathways (2, 3, 8, 14, 18, 19, 37). It has been proposed that exoenzyme S might contribute to pathogenesis by promoting the dissemination of *P. aeruginosa* from the site of infection, due to damage to local host defenses (29). The disruption of normal vesicle trafficking due to ADP-ribosylation of low-molecular-weight GTP-binding proteins might decrease the antimicrobial functions of cells such as neutrophils and macrophages and thereby contribute to dissemination. In addition, ADP-ribosylation catalyzed by exoenzyme S might aid in the identification of proteins that normally interact with p21^{ras} and related proteins in the cell.

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