Ecto-ADP-Ribosyltransferase Activity of *Pseudomonas aeruginosa* Exoenzyme S

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*Pseudomonas aeruginosa* produces two ADP-ribosyltransferases, exotoxin A and exoenzyme S (ExoS). Although the physiological target protein remains to be defined, ExoS has been shown to ADP-ribosylate several eukaryotic proteins in vitro, including vimentin and members of the family of low-molecular-weight GTP-binding proteins. Recently, ExoS ADP-ribosyltransferase activity has been detected in the pleural fluid of rabbits infected with *P. aeruginosa*. This observation prompted an examination of the potential for ExoS to function as an ecto-ADP-ribosyltransferase. We have observed that ExoS preferentially ADP-ribosylates two extracellular serum proteins with molecular masses of 150 and 27 kDa. The ADP-ribosylation of these serum proteins by ExoS was stimulated by, but not dependent upon, exogenous FAS (for factor activating exoenzyme S), which indicated that serum contained endogenous FAS activity. Biochemical analysis showed that the 150-kDa ADP-ribosylated protein was immunoglobulin of the immunoglobulin G (IgG) and IgA classes. Subtyping showed that ExoS preferentially ADP-ribosylated human IgG3 and that ADP-ribosylation occurred within its Fc region. The 27-kDa protein ADP-ribosylated by ExoS was determined to be apolipoprotein A1. These data demonstrate ecto-ADP-ribosyltransferase activity by ExoS. This may extend the potential physiological consequences of ExoS during infection by *P. aeruginosa* beyond the implicated type III secretion-mediated intracellular delivery of ExoS into sensitive eukaryotic cells.

Neutropenia, cystic fibrosis, and burn wounds predispose individuals to infection by *P. aeruginosa* (4). *P. aeruginosa* produces several extracellular virulence factors (28), including exotoxin A and exoenzyme S (ExoS), which are members of the family of bacterial ADP-ribosyltransferases (14). Relative to other family members, ExoS possesses several unique biochemical properties. First, while most bacterial ADP-ribosyltransferases target specific host proteins for ADP-ribosylation, exoenzyme S has been shown to ADP-ribosylate several host proteins in vitro, including vimentin (6) and several low-molecular-weight GTP-binding proteins, including p21ras (8). It should be noted that the in vivo target protein(s) of exoenzyme S is undefined. Second, exoenzyme S possesses an absolute requirement for an eukaryotic protein, termed FAS (for factor activating exoenzyme S), to catalyze the ADP-ribosylation reaction (7). Third, while most bacterial ADP-ribosyltransferases possess a recognized A:B structure-function organization (14), the A:B organization of exoenzyme S is not apparent.

*P. aeruginosa* secretes exoenzyme S as a high-molecular-weight aggregate composed of two proteins with molecular masses of 53 and 49 kDa (5, 26). The 53- and 49-kDa forms of exoenzyme S are encoded by separate genes, termed *exoS* (encoding ExoS) and *exsA* (encoding ExoS), respectively (17). Although Exo53 and ExoS possess 75% primary amino acid homology, Exo53 catalyzes the ADP-ribosylation of target proteins at only 0.2% of the rate of ExoS (37). The ADP-ribosyltransferase domains of ExoS3 and ExoS6 have been located within their carboxyl termini, and both Exo53 and ExoS6 have been shown to possess a glutamic acid which is required for expression of catalytic activity (20).

Recent efforts have focused on defining the physiological intracellular target protein(s) of exoenzyme S and the physiological consequence of ADP-ribosylation. Other studies have identified ExoS ADP-ribosyltransferase activity in extracellular compartments, such as blood (plasma) and pleural fluid (15), during infection of animal models by *P. aeruginosa* and more recently in the tissue culture media of eukaryotic cells cocultured with *P. aeruginosa* (29). These observations, along with the recent recognition that eukaryotes possess endogenous ecto-ADP-ribosyltransferases (13), prompted the present study to determine the capacity of ExoS to act as an ecto-ADP-ribosyltransferase. Our studies have identified two extracellular proteins which appear to be preferentially ADP-ribosylated by ExoS, apolipoprotein A1 (ApoA1) and immunoglobulin. In addition, we have observed extracellular FAS activity.

**MATERIALS AND METHODS**

Materials. Reagents were purchased from Sigma unless otherwise stated. [*32P-adenylate phosphate*]NAD was purchased from Dupont-New England Nuclear. Recombinant FAS was a gift from H. Fu (Emory University) and R. J. Collier (Harvard Medical School). Exoenzyme S was purified from *P. aeruginosa* PAK as previously described (16). Bovine serum albumin was used as a standard to normalize protein concentrations and was purchased from Pierce Biochemical.

ADP-ribosylation of serum proteins. Reaction mixtures (50 μl) contained 10% serum (bovine or human) in Dulbecco’s phosphate-buffered saline, 6 μM [*32P-adenylate phosphate*]NAD (specific activity, 2 × 10^6 cpm/150 pmol), 1.0 mM ADP-ribose, and 0.2 μM *P. aeruginosa* 388 exoenzyme S with or without 0.2 μM recombinant FAS. Reactions were performed for 1 h at either room temperature or 37°C and stopped with 0.5 volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer with or without β-mercaptoethanol, and the reaction mixtures were boiled for 5 min. SDS-PAGE was performed as described by Laemmli (18) with resolving gels of 10 or 13.5% acrylamide and 3% stacking gel. Gels were stained, dried, and subjected to autoradiography, followed by scintillation counting.

ADP-ribosylation of pleural fluid proteins. Reaction mixtures (25 μl) contained 5 μl of rabbit pleural fluid infected with *P. aeruginosa* PAK (exoS) or *P. aeruginosa* PAK exsA::omega (exoS) and 3 μM [*32P-adenylate phosphate*]NAD (specific activity, 2 × 10^6 cpm/150 pmol), with or without 24 nM *P. aeruginosa* 388 exoenzyme S. Note that these assays were performed in the absence of exogenous FAS. Reactions were performed for 0.5, 1, 2, or 3 h at room temperature and stopped with 0.5 volume of SDS-PAGE loading buffer without β-mercaptop...
inhibitor [SBTI] at 2 to 10 ADP-ribose, the target protein (immunoglobulin, ApoA1, or soybean trypsin exoenzyme S did preferentially ADP-ribosylate two proteins in the reaction mix are indicated above the appropriate lane. Reaction mixtures were subjected to SDS-PAGE with (Reduced) or without (Oxidized) β-mercaptoethanol. Gels were stained for total protein with Coomassie blue (left panel) and then subjected to autoradiography (right panel). Molecular mass markers (with sizes in kilodaltons) are in the leftmost lane.

toethanol, and the reaction mixtures were boiled for 5 min. SDS-PAGE and autoradiography were then performed.

ADP-ribosylation of purified proteins. Reaction mixtures (25 μl) contained 0.2 M sodium acetate (pH 6.0), 33 or 100 μM [32P]-adenylate-phosphate]NAD (specific activity, 2 × 10⁶ cpm/75 or 250 pmol), 36 mM recombinant FAS, 1.0 mM ADP-ribose, the target protein (immunoglobulin, ApoA1, or soybean trypsin inhibitor [SBTI] at 2 to 10 μM), and 14 mM ΔN222, ΔN222 was digested in 25 mM Tris (pH 7.6) containing 0.1 mg of egg albumin/ml. Incubation was for between 20 and 240 min at room temperature or 37°C. Reactions were stopped by the addition of 0.5 volume of gel loading buffer with or without β-mercaptoethanol, which was followed by SDS-PAGE and autoradiography. The radiolabel was quantitated by scintillation counting.

Amino-terminal amino acid sequencing of human ApoA1. Human serum was subjected to SDS–12% PAGE without β-mercaptoethanol and electrophorized to a polyvinylidene difluoride membrane. Following amido black staining, the 27-kDa band was excised and subjected to amino-terminal protein sequencing as described previously (2).

Purification of histidine fusion proteins. Histidine fusion proteins were expressed in Escherichia coli essentially as described by Novagen. Protease-sensitive proteins were purified in the presence of a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin), which were added to Escherichia coli conditioned culture medium, which indicated that these proteins were components of the culture medium (newborn calf serum) and not derived from the CHO cells. Exoenzyme S appeared to ADP-ribosylate the same proteins, with respect to apparent molecular mass, in human sera as observed for newborn calf serum (Fig. 1). Under reducing conditions, the electrophoretic migration of the 150-kDa radiolabeled band shifted to several radiolabeled bands which migrated with apparent molecular masses of between 50 and 65 kDa. In contrast, the electrophoretic migration of the 27-kDa radiolabeled band did not change with the addition of the reducing agent. Exoenzyme S ADP-ribosylated both the 150- and 27-kDa proteins in seven independent sera (data not shown). The ADP-ribosylation of the 150- and 27-kDa proteins by ExoS was stimulated by, but not dependent upon, exogenous FAS (Fig. 1). This indicated that serum contained limiting amounts of a FAS-like protein. Under identical assay conditions, ExoS did not catalyze the ADP-ribosylation of serum proteins (data not shown).

ADP-ribosylation of immunoglobulin by ExoS. The shift in apparent molecular mass of the 150-kDa protein ADP-ribosylated by exoenzyme S to several radiolabeled proteins with apparent molecular masses between 50 and 65 kDa during electrophoresis under reducing conditions was consistent with the radiolabeled protein being the heavy chain of immunoglobulin. This prompted the determination of whether exoenzyme S could ADP-ribosylate pooled fractions of immunoglobulin. Exoenzyme S more efficiently ADP-ribosylated purified human immunoglobulin G (hIgG) and IgA than IgM, with the heavy chain of each immunoglobulin acting as the preferred target (Fig. 2). The stoichiometry (moles of ADP-ribose incorporated per mole of heavy chain) for the ADP-ribosylation of each class of immunoglobulin was 0.1 for IgG, 0.1 for IgA, and 0.01 for IgM.

Subtyping of hIgG as a target for ADP-ribosylation identified hIgG3 as the preferred subtype. Figure 3 shows that hIgG3 was ADP-ribosylated at a higher rate and to a greater level than the other hIgG subtypes. The stoichiometry (moles of ADP-ribose incorporated per mole of heavy chain) was 0.1 for the hIgG pool, 0.1 for hIgG1, 0.2 for hIgG2, 0.5 for hIgG3, and 0.02 for hIgG4. Figure 2 also shows that the heavy chains of hIgG2 and hIgG3 were preferentially ADP-ribosylated by exoenzyme S.

ADP-ribosylated rabbit IgG was subjected to papain diges-

RESULTS

ADP-ribosylation of serum proteins by exoenzyme S. The ability of exoenzyme S to ADP-ribosylate extracellular proteins was assayed on cultured CHO cells. Although the ADP-ribosylation of surface proteins on CHO cells was not detected, exoenzyme S did preferentially ADP-ribosylate two proteins in the conditioned culture medium, with apparent molecular masses of 150 and 27 kDa when analyzed by SDS-PAGE under nonreducing conditions (data not shown). Exoenzyme S ADP-ribosylated both proteins with equal efficiency in either fresh or conditioned culture medium, which indicated that these proteins were components of the culture medium (newborn calf serum) and not derived from the CHO cells. Exoenzyme S appeared to ADP-ribosylate the same proteins, with respect to apparent molecular mass, in human sera as observed for newborn calf serum (Fig. 1). Under reducing conditions, the electrophoretic migration of the 150-kDa radiolabeled band shifted to several radiolabeled bands which migrated with apparent molecular masses of between 50 and 65 kDa. In contrast, the electrophoretic migration of the 27-kDa radiolabeled band did not change with the addition of the reducing agent. Exoenzyme S ADP-ribosylated both the 150- and 27-kDa proteins in seven independent sera (data not shown). The ADP-ribosylation of the 150- and 27-kDa proteins by ExoS was stimulated by, but not dependent upon, exogenous FAS (Fig. 1). This indicated that serum contained limiting amounts of a FAS-like protein. Under identical assay conditions, ExoS did not catalyze the ADP-ribosylation of serum proteins (data not shown).

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ADP-ribosylated rabbit IgG was subjected to papain diges-
tion and subsequent SDS-PAGE and autoradiography to localize the site of ADP-ribosylation. Under these conditions, the radiolabel migrated with the Fc region of immunoglobulin (data not shown). The ability of exoenzyme S to ADP-ribosylate purified rabbit Fc was then assayed. Under these conditions, ExoS ADP-ribosylated Fc to a similar extent as SBTI (Fig. 4). The ADP-ribosylated low-molecular-weight band in the Fc preparation, seen in Fig. 4, appears to be a degradation product of Fc.

Protein A, which interacts with the Fc portion of immunoglobulin, blocked the ADP-ribosylation of rabbit IgG by ExoS (Fig. 5). These data were consistent with the ADP-ribosylation of immunoglobulin by ExoS occurring within the Fc region. Other experiments showed that ADP-ribosylated rabbit IgG retained the ability to bind protein A-Sepharose (data not shown). This indicated that the ability of protein A to inhibit the ADP-ribosylation of rabbit IgG was probably due to a steric inhibition of the interaction between ExoS and the immunoglobulin molecule and that the protein A binding site within the immunoglobulin molecule is near, but is not, the absolute site of ADP-ribosylation.

**ADP-ribosylation of ApoA1 by ExoS.** The identity of the 27-kDa human serum protein that was ADP-ribosylated by ExoS was determined (Fig. 1). This protein did not appear to be immunoglobulin light chain, since its apparent molecular mass was constant during SDS-PAGE under both reducing and nonreducing conditions. To facilitate identification, the 27-kDa human serum protein was subjected to protein sequencing. The amino-terminal 10 residues were determined to be NH₂-DEPPQSPXDRCOOH. A FASTA search of the Swiss-Prot protein database identified this sequence as the amino terminus of ApoA1. ApoA1 is a human lipid-binding protein stable in sodium chloride and mercuric chloride (Table 1). The ADP-ribose linkages to ApoA1 and rabbit Fc formed by ExoS were shown to be consistent with an arginine linkage (8). The ADP-ribose linkage possesses a characteristic chemical sensitivity. The chemical sensitivity of p21c-Hras ADP-ribosylated purified human ApoAI was then determined. ExoS ADP-ribosylated authentic ApoA1 in a FAS- and time-dependent manner. The stoichiometry (moles of ADP-ribose incorporated per mole of ApoA1) of ADP-ribosylation was about 0.5.

**Characterization of the ADP-ribose linkage to ApoAI and IgG.** Bacterial ADP-ribosylating exotoxins covalently modify specific amino acids within the target protein, including Arg, Cys, Asn, and diphthamide (1). Each ADP-ribose–amino acid linkage possesses a characteristic chemical sensitivity. The chemical sensitivity of p21c-Hras ADP-ribosylated by ExoS was shown to be consistent with an arginine linkage (8). The ADP-ribose linkages to ApoA1 and rabbit Fc formed by ExoS were sensitive to hydroxylamine and sodium hydroxide but were stable in sodium chloride and mercuric chloride (Table 1). These data are consistent with ApoA1 and Fc being ADP-ribosylated by ExoS at an arginine residue (30).

**Exoenzyme S activity in the pleural fluid of rabbits infected with P. aeruginosa.** ExoS activity has been observed within the pleural fluid of rabbits infected with *P. aeruginosa* (15); we have confirmed this observation. A more detailed analysis showed that the ExoS present in the pleural fluid of rabbits infected with *P. aeruginosa* PAK ADP-ribosylated specific pleural fluid proteins, with one of the ADP-ribosylated pro-
mice but was less efficient at spreading to the bloodstream than the parental strain. These results correlated tissue damage and dissemination with an ExsA-regulated protein (25–27). While it is attractive to implicate ExoS as the mediator for the defective properties of this mutant, the defect in the pathological properties of this mutant may be attributed to other ExsA-regulated proteins. Indeed, Fleischig et al. (9) reported that in an acute-cytotoxicity model, P. aeruginosa PA103 yielded a cytotoxic phenotype which did not correlate with ExoS production. However, it should be noted that ExoS may contribute to the intoxication process of P. aeruginosa at a step that was not a component of this model system.

The earlier observations that ExoS was detected in extracellular environments (3, 15) prompted the determination of whether ExoS preferentially ADP-ribosylated extracellular proteins. In this regard, we observed that ExoS catalyzed the ADP-ribosylation of both immunoglobulin and ApoA1. Although it is difficult to predict the physiological significance of the ADP-ribosylation of these extracellular proteins, the ability to modify extracellular target proteins appears to expand the capacity of ExoS to contribute to the pathogenesis of P. aeruginosa. Individuals afflicted with cystic fibrosis frequently develop pulmonary complications as a result of chronic P. aeruginosa infections. High levels of complement activation capacity in sera from cystic fibrosis patients correlate with high levels of hlgG3 antibodies to P. aeruginosa antigens and poor lung function (32). Thus, it is possible that the ADP-ribosylation of hlgG3 modulates a function of the immunoglobulin, which could influence bacterial dissemination and/or tissue damage. ApoA1, another extracellular protein that was ADP-ribosylated by ExoS, is normally found associated with high-density lipoprotein particles in plasma, where it participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase.

Future studies will determine if ADP-ribosylation of extracellular target proteins occurs in vivo during P. aeruginosa infections. This may help resolve whether ExoS-mediated ADP-ribosylation of extracellular proteins is a general property of ExoS or whether the ADP-ribosylation of extracellular target proteins is mediated as a local event, such as at the site of tissue destruction, where one would expect to find enhanced pools of extracellular NAD. The determined concentration of NAD in various mammalian plasmas has been reported to be between 0.1 and 0.3 μM (11), which is below the observed K_m of ExoS for NAD (12).

The FAS protein is a member of the family of 14-3-3 proteins, which possess several cellular functions, including the regulation of exocytosis and the modulation of the activities of protein kinase C and tyrosine and tryptophan hydroxylases (19). The recent observations that 14-3-3 proteins interact with intracellular signaling proteins, including Raf (10, 33), have

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**DISCUSSION**

Earlier genetic analysis implicated an ExsA-regulated protein as a mediator of P. aeruginosa pathogenesis in burn wounds and chronic lung infections (24). A PscC mutant (ExsA^-) (388exs1::Tn1) did not express ExoS activity and was 2,000-fold less virulent than the parental strain (strain 388) in a mouse burn infection model (24, 38). This mutant colonized mice but was less efficient at spreading to the bloodstream than the parental strain. These results correlated tissue damage and dissemination with an ExsA-regulated protein (25–27). While it is attractive to implicate ExoS as the mediator for the defective properties of this mutant, the defect in the pathological properties of this mutant may be attributed to other ExsA-regulated proteins. Indeed, Fleischig et al. (9) reported that in an acute-cytotoxicity model, P. aeruginosa PA103 yielded a cytotoxic phenotype which did not correlate with ExoS production. However, it should be noted that ExoS may contribute to the intoxication process of P. aeruginosa at a step that was not a component of this model system.

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increased interest in understanding the biology of the 14-3-3 proteins. Our studies have identified an FAS-like activity in serum and pleural fluid, two extracellular environments. Dara Frank (9a) has also observed FAS activity in pleural fluid. Earlier reports (19) indicated that several members of the 14-3-3 family may be secreted from eukaryotic cells. Thus, it is possible that 14-3-3 proteins may be components of extracellular environments, which may extend their regulatory capacity.

The feasibility of ExoS functioning as an extracellular ADP-ribosyltransferase is supported by an increased awareness that extracellular eukaryotic enzymes metabolize NAD. Earlier studies reported the presence of extracellular NADases on eukaryotic target proteins for ADP-P. aeruginosa secreted from host cells (13, 15). Presently, ExoS has been shown to ADP-ribosylate both intra- and extracellular eukaryotic target proteins (13, 15). Thus, it is possible that ExoS targets both intra- and extracellular eukaryotic target proteins for ADP-ribosylation.

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