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J. T. Barbieri · J. Sun

Pseudomonas aeruginosa ExoS and ExoT

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Abstract ExoS and ExoT are bi-functional type-III cytotoxins of *Pseudomonas aeruginosa* that share 76% primary amino acid homology and contain N-terminal RhoGAP domains and C-terminal ADP-ribosylation domains. The Rho GAP activities of ExoS and ExoT appear to be biochemically and biologically identical, targeting Rho, Rac, and Cdc42. Expression of the RhoGAP domain in mammalian cells results in the disruption of the actin cytoskeleton and interference of phagocytosis. Expression of the ADP-ribosyltransferase domain of ExoS elicits a cytotoxic phenotype in cultured cells, while expression of ExoT appears to interfere with host cell phagocytic activity. Recent studies showed that ExoS and ExoT ADP-ribosylate different substrates. While ExoS has poly-substrate specificity and can ADP-ribosylate numerous host proteins, ExoT ADP-ribosylates a more restricted subset of host proteins including the Crk proteins. Protein modeling predicts that electrostatic interactions contribute to the substrate specificity of the ADP-ribosyltransferase domains of ExoS and ExoT.

Abbreviations *GAP:* GTPase activating protein · *ADP-r:* Adenosine diphosphate ribose · *MLD:* Membrane localization domain · *FAS:* Factor activating exoenzyme S · *Crk:* CT-10 regulator of kinase · *SBTI:* Soybean trypsin inhibitor · *GEF:* Guanine nucleotide exchange factor

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen and the most common Gram-negative bacterium associated with nosocomial infections. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections (Van

J. T. Barbieri (✉) · J. Sun
Microbiology and Molecular Genetics, Medical College of Wisconsin,
8701 Watertown Plk. Road, Milwaukee, WI, 53226, USA
e-mail: jtb01@mcw.edu · Tel.: +1-414-4568412 · Fax: +1-414-4566535

Delden and Iglewski 1998). Immunocompromised individuals, such as patients with cystic fibrosis, neutropenic cancer, burn wound, and bone marrow transplantation, are susceptible to infection by this pathogen and the prevalence of multi-drug resistant strains limits antibiotic therapy. *P. aeruginosa* pathogenesis depends on cell associated and secreted virulence factors. Cell-associated virulence factors include flagellum, pilus, adhesins, alginate, and lipopolysaccharide. Secreted virulence factors include: hemolysins, lipases, proteases, Exotoxin A and the type-III cytotoxins; ExoS, ExoT, ExoU, and ExoY.

The type-III secretion system delivers cytotoxins directly from the bacterium into a mammalian cell through a contact-dependent mechanism (Blocker et al. 2001). The type-III secretion apparatus is composed of 20–30 proteins that are conserved among Gram-negative bacteria. The genes encoding the *Pseudomonas* type-III secretion system are clustered in the 55 min region of the *P. aeruginosa* PA01 chromosome and are designated *psc*, *pcr*, *exs*, and *pop*. *psc* and *pcr* encode proteins of the type-III secretion apparatus and proteins involved in the regulation of expression of the system. In addition, PopB and PopD are responsible for the translocation of the type-III cytotoxins into mammalian cells (Frank 1997).

Pseudomonas aeruginosa type-III cytotoxins inhibit host innate immunity. ExoU is a phopholipase (Sato et al. 2003) and is correlated with acute cytotoxicity in epithelial cells and macrophages, and contributes to injury in models systems (Finck-Barbancon et al. 1997; Vallis et al. 1999). ExoY is an adenylate cyclase, which elevates the intracellular cAMP levels in cultured mammalian cells and causes actin cytoskeleton reorganiza tion (Yahr et al. 1998). ExoS and ExoT are similar, yet distinct, possessing N-terminal RhoGAP and C-terminal ADP-ribosyltransferase domains. The RhoGAP domains of ExoS and ExoT stimulate the reorganization of actin cytoskeleton through inactivation of Rho GTPases, while the ADP-ribosyltransferase domains ADP-ribosylate distinct host proteins.

This review will discuss the structural and biochemical properties of ExoS and ExoT and their relative roles in *P. aeruginosa* pathogenesis.

History of *Pseudomonas aeruginosa* exoenzyme S

Exoenzyme S was first described as a secreted ADP-ribosyltransferase that was isolated from *P. aeruginosa* strain 388, possessing properties distinct from Exotoxin A (Iglewski et al. 1978). Exoenzyme S did not ADP-ribosylate elongation factor 2, as did Exotoxin A and diphtheria toxin, but ADP-ribosylated several proteins present in crude extracts of wheat germ or rabbit reticulocytes. In addition, the ADP-ribosyltransferase activity of exoenzyme S was not neutralized by α-Exotoxin A antibody and relative to Exotoxin A was heat stable. Together, these properties indicated that exoenzyme S was different from the previously described Exotoxin A.

From the culture supernatant fluid of *P. aeruginosa* strain 388, two proteins with molecular weight 53-kDa and 49-kDa (termed the 53- and 49-kDa forms of exoenzyme S) copurified with the ADP-ribosyltransferase activity of exoenzyme S (Kulich et al. 1993). The 53- and 49-kDa forms of exoenzyme S had similar N-terminal amino acid sequences (Coburn 1992), immunoreactivity (Nicas and Iglewski 1984), and peptides upon proteolytic cleavage (Kulich et al. 1994). Upon fractionation, the purified 49-kDa form of exoenzyme S possessed ADP-ribosylation activity, while the 53-kDa form lacked ADP-ribosylation activity. α-49-kDa-protein IgG inhibited the ADP-ribosyltransferase activity of purified exoenzyme S in a dose-dependent manner. This suggested that the 53-kDa form of

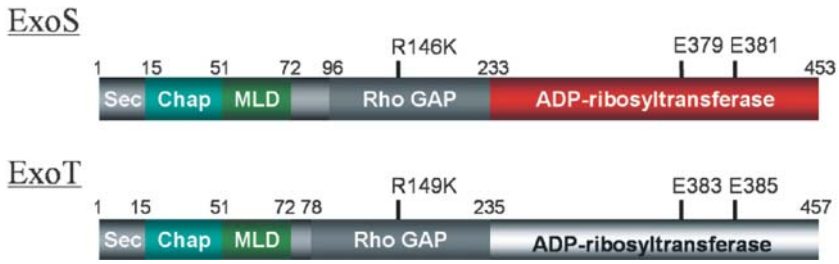

Fig. 1 Functional domains of *Pseudomonas aeruginosa* ExoS and ExoT. ExoS and ExoT are similar yet distinct. They share 76% amino acid identity and similar functional domains with N-terminal RhoGAP activity and C-terminal ADP-ribosyltransferase activity. The N terminus of ExoS and ExoT are similar, containing secretion signals (*Sec*), chaperon binding domains (*Chap*), membrane localization domains (*MLD*) and RhoGAP domains (*RhoGAP*). The RhoGAP domains of ExoS and ExoT stimulate reorganization of the actin cytoskeleton through inactivating Rho, Rac and Cdc42. R146 of ExoS and R149 of ExoT are catalytic residues of the RhoGAP activity. ExoS and ExoT have distinct C-terminal ADP-ribosylation domains

exoenzyme S was an inactive precursor of the 49-kDa form of exoenzyme S. Subsequent studies showed that the 53- and 49-kDa forms of exoenzyme S are encoded by distinct genes, termed *exoT* and *exoS*, respectively (Yahr et al. 1996).

Functional domains of ExoS and ExoT

ExoS and ExoT share 76% amino acid identity (Fig. 1) and are bi-functional type-III cytotoxins with RhoGAP domains, which inactivate Cdc42, Rac and Rho, and C-terminal ADP-ribosyltransferase domains, which ADP-ribosylate unique cellular host proteins. The N termini of ExoS and ExoT also comprise a signal sequence required for secretion through the type-III apparatus, a chaperone binding region, and a membrane localization domain (MLD). The MLD, a hydrophobic region that is enriched with leucines, localizes ExoS and ExoT to intracellular membranes after translocation into host cells through the type-III secretion system.

Membrane localization domain

The MLD was first determined to be N-terminal to the GAP domain (Pederson et al. 2000), which resided within residues 96–219 of ExoS. The MLD was not required for expression of ExoS RhoGAP in cultured cells. Subsequent studies mapped the MLD to residues 51–72 of ExoS, and observed that these residues were necessary and sufficient for membrane localization within mammalian cells (Pederson et al. 2002). A deletion protein of ExoS that lacked the MLD (ExoS(ΔMLD) could be secreted through the type-III secretion system of ExoS and translocated into mammalian cells. This indicated that the MLD was not required for secretion through the type-III apparatus or translocation into mammalian cells. Although ExoS(ΔMLD) ADP-ribosylated Ras in vitro, type-III delivered ExoS(ΔMLD) did not ADP-ribosylate Ras, a membrane-associated target protein of ExoS (Riese and Barbieri 2002). This indicated that the subcellular localization of ExoS contributed to the targeting of Ras and other host proteins. Type III-delivered ExoS(ΔMLD)

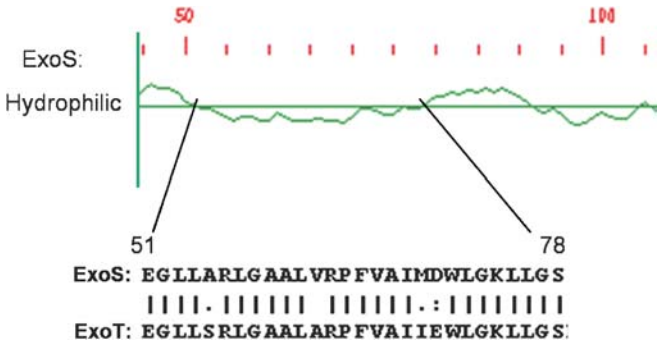

Fig. 2 Membrane localization domains of *Pseudomonas aeruginosa ExoS and ExoT. Upper panel:* the hydrophilic plot of N-terminal ExoS (residues *50–100*). *Lower panel:* the sequence alignment of the membrane localization domains of ExoS (*51–78*) and ExoT (*51–78*)

and ExoS showed similar capacities to elicit a cytotoxic response in CHO cells, which uncoupled the ADP-ribosylation of Ras from the cytotoxic action of ExoS (Riese and Barbieri 2002). Subsequently, the N terminus of *Yersinia* YopE was also shown to possess an intracellular membrane localization domain and that the MLD of YopE colocalized with the MLD of ExoS within mammalian cells and that the MLD of YopE functionally complemented the ExoS MLD for intracellular targeting in mammalian cells (Krall et al. 2004).

While the membrane localization domain of ExoT has not been experimentally mapped, primary amino acid sequences between the MLD of ExoS and the analogous region of ExoT are highly homologous (Fig. 2), indicating that ExoT possesses a similar targeting sequence to the ExoS MLD. This is supported by the determination that the type-III-delivered ExoT has a similar intracellular fractionation pattern with ExoS and that the RhoGAP domains of ExoS and ExoT have the same eukaryotic targets, Rho, Rac, and Cdc42 (Kazmierczak and Engel 2002; Krall et al. 2002). While the ADP-ribosylation domains of ExoS and ExoT have different in vivo target specificity, switching the ADP-ribosylation domains of ExoS and ExoT switched the in vivo substrate specificity of ExoS and ExoT. This suggests that the MLDs of ExoS and ExoT target these two toxins to a common intracellular trafficking pathway, where the intrinsic biochemical properties of the toxins become the only determinant for their different target specificity (Sun and Barbieri 2004).

RhoGAP domains

When ExoS was expressed in *Yersinia*, a mutated form of ExoS with deficient ADP-ribosyltransferase activity retained partial ability to disrupt the actin cytoskeleton in host cells and was resistant to phagocytosis, indicating ExoS could act through two different mechanisms on host cells (Frithz-Lindsten et al. 1997). Subsequently, the intracellular expression of the N-terminal 234 amino acids of ExoS (ExoS91–234) was observed to elicit the rounding of Chinese Hamster Ovary (CHO) cells and to disrupt the actin cytoskeleton. Cytoskeleton rearrangement elicited by ExoS(1–234) was reversed by the addition of cytotoxic necrotizing factor 1 (CNF 1), a toxin that constitutively activated Rho proteins

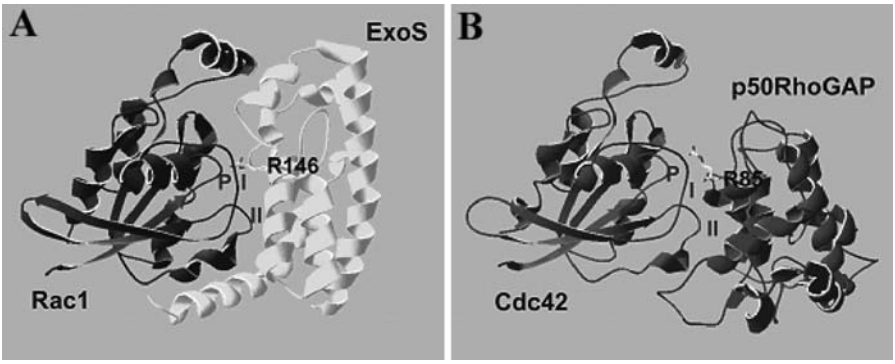

Fig. 3A, B Structures of bacterial GAP (*ExoS*) and eukaryotic GAP (*p50rhoGAP*). The atomic coordinates of Cdc42-p50rhoGAP complex (*1AM4*) and Rac1-ExoS RhoGAP complex (*1HE1*) were downloaded from PDB, and the structures were displayed in Swiss-Pdb-viewer v3.7. **A** Structure of Rac1-ExoS RhoGAP complex. Switch I (*I*), Switch II (*II*), and P-loop (*P*) in Rac1 are shown. The catalytic residue R146 in ExoS is shown. **B** Structure of Cdc42-p50RhoGAP complex. Switch I (*I*), Switch II (*II*), and P-loop (*P*) in Cdc42 are shown. The catalytic residue R85 in p50RhoGAP is shown

through deamidation of Gln61/63, which implicated a role for Rho GTPases in the disruption of the actin cytoskeleton by ExoS (Pederson et al. 1999). ExoS(1–234) was then shown to be a Rho GTPase Activating Protein (RhoGAP) for Rho, Rac, and Cdc42 and that Arg146 was a catalytic residue required for the expression of RhoGAP activity (Goehring et al. 1999). The 3-D crystal structure of the Rac-ExoSGAP complex showed that although ExoSGAP and the mammalian RhoGAPs did not share structural homology, like the eukaryotic RhoGAPs, ExoS RhoGAP stabilized the transition state of GTPase reaction, indicating ExoS RhoGAP was a functional mimic of eukaryotic GAPs (Wurtele, Renault et al. 2001; Wurtele, Wolf et al. 2001) (Fig. 3).

Similar to ExoS, the N terminus of ExoT is a Rho GAP for Rho, Rac, and Cdc42 in vitro and in vivo (Krall et al. 2000; Kazmierczak and Engel 2002), and that Arg149 (analogous to Arg146 on ExoS) is required for the Rho GAP activity of ExoT (Garrity-Ryan et al. 2000; Geiser et al. 2001). The function of ExoT Rho GAP activity has been proposed to be the inhibition of phagocytosis of bacterium by polarized epithelial cells and macrophage-like cells (Garrity-Ryan et al. 2000).

ADP-ribosyltransferase domains

ExoS was first described as an ADP-ribosyltransferase activity that was distinct from Exotoxin A, catalyzing the transfer of ADP-ribose from NAD^+ to a number of unknown eukaryotic proteins, but not EF-2 (Iglewski et al. 1978). The intermediate filament protein vimentin was first identified as a cellular target of ExoS (Coburn, Dillon et al. 1989). Ras and several Ras related proteins, including Rab3, Rab4, Ral, Rap1A, and Rap2, were subsequently identified as targets of ExoS (Coburn, Wyatt et al. 1989; Coburn and Gill 1991). The ADP-ribosylation of Arg41 by ExoS disrupted Ras signaling by inhibiting Guanine Nucleotide Exchange Factor (GEF) catalyzed nucleotide exchange, which uncoupled Ras signal transduction (Ganesan et al. 1998, 1999). ExoS also ADP-ribosylated Rap at Arg41, which also inhibited the ability of Rap GEF, C3G, to stimulate nucleotide exchange (Riese

et al. 2001). Type-III-delivered ExoS was found to be auto-ADP-ribosylated, which may regulate the toxin's catalytic potential (Riese et al. 2002). ExoS ADP-ribosyltransferase activity was dependent on a mammalian 14–3-3 protein termed *F*actor *A*ctivating Exo*S* (FAS) (Coburn et al. 1991; Fu et al. 1993). While FAS directly bound ExoS and Raf, activation of the ADP-ribosylation activity of ExoS by FAS appeared complex (Zhang et al. 1997, 1999; Petosa et al. 1998; Henriksson et al. 2000). The ADP-ribosyltransferase activity of ExoS was mapped to C-terminal residues 234–453 (Knight et al. 1995), where Glu 381 was determined to be a catalytic residue in the ADP-ribosylation reaction. The E381D mutation of ExoS reduced ADP-ribosyltransferase and NAD glycohydrolase activities. In contrast, the E379D mutation did not affect NAD glycohydrolase active, but inhibited ADP-ribosyltransferase activity, indicating that Glu379 contributed to the transfer of ADP-ribose to the target proteins (Liu et al. 1996; Radke et al. 1999). This identified ExoS as a bi-glutamic acid transferase.

While the C terminus of ExoT also has a FAS-dependent ADP-ribosyltransferase activity, it only possesses ~0.1% of ADP-ribosyltransferase activity relative to ExoS when soybean trypsin inhibitor (SBTI) is used as an artificial substrate. Interestingly, ExoT only possessed ~2% NAD glycohydrolase activity of ExoS (Liu et al. 1997). Intracellular expression of ExoS in the ADP-ribosylation domain was cytotoxic to culture cells, but ExoT was not cytotoxic to cultured cells (Note: the term "cytotoxicity" in the present review is defined as the ability of toxins to cause cell death). This implied that ExoT was a defective ADP-ribosyltransferase. However, recent studies observed that ExoT elicited reorganization of the actin cytoskeleton without interfering with Ras signal transduction, and the mutated form of ExoT (ExoTR149A) that was deficient in Rho GAP activity caused a morphological change of infected HeLa cells, suggesting that ExoT may ADP-ribosylate unique host proteins, distinct from Ras (Sundin et al. 2001). The observation that bacterial strains carrying point mutations at Arg149 of ExoT were internalized to an intermediate extent, compared to strains with a deletion of ExoT versus strains expressing wild-type ExoT, also suggested that the Rho GAP activity of ExoT accounted for only part of the anti-internalization activity (Garrity-Ryan et al. 2000). This implied that the ADP-ribosyltransferase domain of ExoT was active. Subsequent studies showed that ExoT ADP-ribosylated host proteins that were distinct to ExoS, Crk-I and Crk-II (CT10-regulator of kinase) both in vitro and in vivo. ExoT ADP-ribosylated Crk at a rate similar to the rate of ADP-ribosylation of SBTI by ExoS (Sun and Barbieri 2003). Crk proteins are SH2-SH3 domain-containing adaptor proteins that play an essential role in integrin-mediated phagocytosis and focal adhesion. Therefore, the ADP-ribosylation of the Crk proteins linked the antiphagocytic activity of ExoT to a mechanism distinct from the modulation of Rho GTPases by the RhoGAP domain. This leads to a hypothesis that ExoT ADP-ribosylates Crk proteins to block Rap1- and Rac1-mediated focal adhesion and phagocytosis.

Current models predict that the ADP-ribosylation of Crk by ExoT could either block interaction with upstream focal adhesion proteins or block interaction with downstream factor DOCK180, which would inhibit Rac-1-mediated phagocytosis (Fig. 4). Alternatively, ExoT ADP-ribosylation of Crk proteins may not block a physical interaction of Crk with its binding partners, but may result in nonfunctional signal complexes. Future studies will identify the sites of ADP-ribosylation on Crk proteins and investigate the mechanism of ExoT-mediated antiphagocytosis through ADP-ribosylation of Crk proteins.

Crk-mediated phagocytosis and focal adhesion appears to be a common pathway that is modulated by bacteria to either prevent uptake of bacteria by professional phagocytes or facilitate bacterial invasion into mammalian cells. *Yersinia* YopH, a type-III secreted

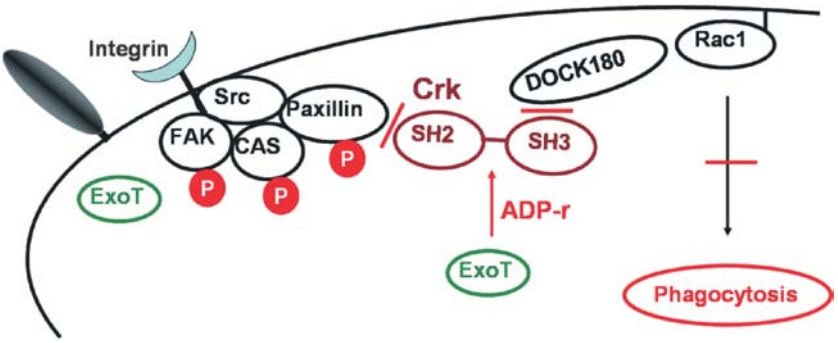

Fig. 4 . ADP-ribosylation of Crk proteins by ExoT inhibits phagocytosis. Once integrin receptors are activated by extracellular stimuli, Src kinase phosphorylates FAK (focal adhesion kinase), Cas (Crk-associated substrate) and Paxillin, which subsequently form a focal adhesion complex on the membrane. The phosphorylated focal adhesion complex recruits Crk proteins to the membrane through interaction with SH2 domain of Crk. The SH3 domain of Crk proteins brings the downstream factor DOCK180 that is the guanine nucleotide exchange factor for Rac1. Activated Rac1 stimulates downstream signal transduction leading to phagocytosis. The type-III delivered ExoT ADP-ribosylates Crk proteins. The ADP-ribosylation of Crk proteins either blocks the upstream interaction between focal adhesion complex and SH2 domain of Crk or blocks the downstream interaction between SH3 domain of Crk with DOCK180 to Rac-mediated phagocytosis

phosphatase, dephosphorylates focal adhesion complex proteins, which downregulate Crk-mediated phagocytosis (Persson et al. 1997; Black et al. 2000). *Shigella flexneri*, an opportunistic intracellular bacterial pathogen, activates Crk-mediated pathways to facilitate invasion. The Abl family of tyrosine kinases (Abl and Arg) is required during *Shigella* internalization. Abl and Arg were reported to be activated during a *Shigella* infection, accumulated at the site of bacterial entry, and were required for efficient bacterial uptake, as internalization was blocked upon deletion of these kinases or treatment with a specific inhibitor. The Abl kinases targeted Crk proteins during *Shigella* uptake, and a phosphorylation-deficient Crk mutant significantly inhibited bacterial uptake. Thus, *Shigella* appears to facilitate bacterial invasion through the modulation of a signal pathway that links Abl kinase phosphorylation of Crk to Rac-mediated phagocytosis (Burton et al. 2003).

Substrate recognition by the ADP-ribosyltransferases of ExoS and ExoT

While sharing 76% amino acid identity, ExoS and ExoT ADP-ribosylate different host proteins (Fig. 5). How ExoS and ExoT recognize different proteins is an intriguing question. Current structural and biochemical studies of bacterial ADP-ribosylating toxins provide insight into the mechanisms of NAD binding, cleavage, and ADP-ribose transfer; however, the mechanism by which bacterial ADP-ribosylating toxins recognize protein substrates is poorly defined. This is partially due to the lack of the structures of enzyme-protein substrate complexes. Recently, the 3-D structures of the ADP-ribosylation domains of ExoS and ExoT were generated by SWISS-MODEL, using VIP2, Iota, and C3 exoenzyme as templates (Fig. 6). Regions B (active site loop), C (ARTT motif), and E (PN loop) on ExoS were necessary and sufficient to recognize ExoS targets, while regions B, C and E on ExoT were necessary, but not sufficient, to recognize ExoT targets, the Crk

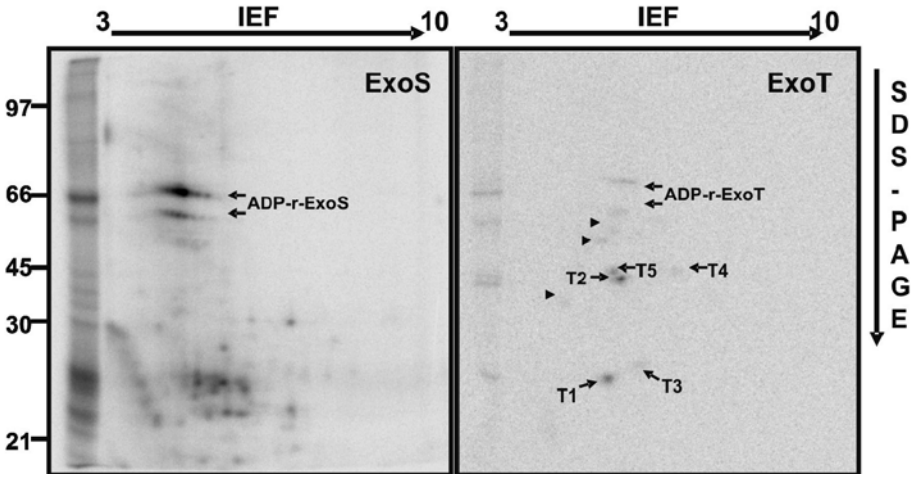

Fig. 5 Type-III delivered ExoS and ExoT ADP-ribosylated unique proteins in CHO cells. Postnuclear supernatant from CHO cells infected with *P. aeruginosa* PA103 Δ*exo*U,*exo*T::Tc expressing ExoS-HA (*ExoS*) or ExoT-HA (*ExoT*) were applied to 2-D SDS-PAGE, (pH 3–10), followed by auto-radiography. Auto-ADP-ribosylated ExoS and ExoT, and proteins that were ADP-ribosylated by ExoT are labeled with indicated *arrows*. Arrows indicate the migration of five radiolabeled spots. Spot T1, T2 and T3 correspond with Crk-I, Crk-II, and PGK-1. (Reproduction from J. Biol. Chem., Vol. 278, 32794–32800, *Pseudomonas aeruginosa* ExoT ADP-ribosylates CT10 Regulator of Kinase (Crk) Proteins. (Jianjun Sun and Joseph T. Barbieri)

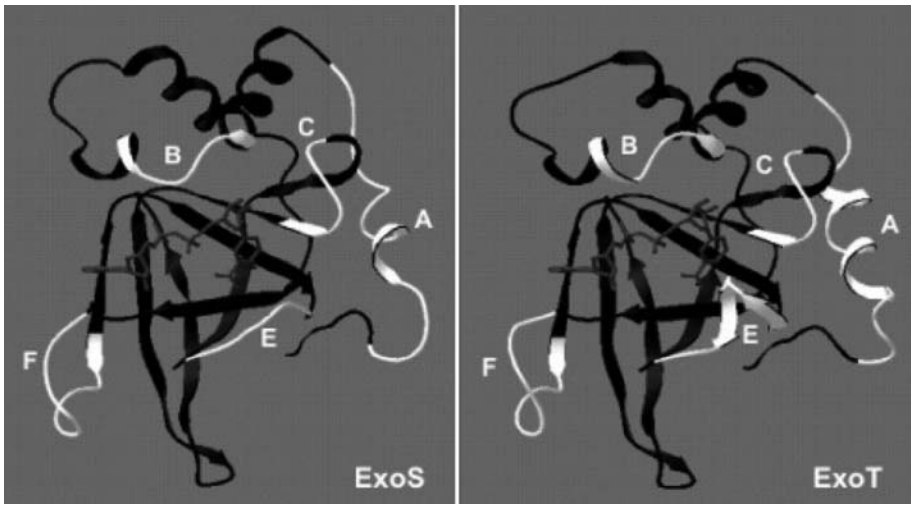

Fig. 6 Protein modeling of the ADP-ribosylation domains of ExoS and ExoT**.** Using the structures of C3 exoenzyme, Vip2 and Iota as templates, the 3-D structures of ExoS and ExoT ADP-ribosylation domains were generated by SWISS-MODEL. The regions that share low homology between ExoS and ExoT are highlighted and labeled as *A*, *B*, *F*, *C*, and *D*, respectively. Note that Regions A, B, C, and E are equivalent to the positions of helix α1, "active site loop," "ARTT" motif, and "PN loop," respectively

proteins. This indicated that an additional region was required for recognition of Crk. Subsequently, a specific Crk recognition motif on ExoT was defined as region A (Helix α1). The calculated electrostatic potentials of the substrate recognition surfaces on ExoS and ExoT displayed significant differences, where ExoS was a mixture of basic, acidic, and neutral charged residues, while ExoT was primarily composed of acidic residues. The electrostatic properties of the substrate recognition surfaces of Ras and Crk are complementary to ExoS and ExoT, respectively (Sun and Barbieri 2004).

ExoS recognizes a wide variety of genetically or structurally diverse proteins for ADP-ribosylation, including the monomeric GTPases, vimentin, and other undefined host proteins. ExoS also undergoes auto-ADP-ribosylation. Even with a one protein substrate, ExoS has multiple alternative sites for ADP-ribosylation. Arg41 of Ras is the preferred site for ExoS ADP-ribosylation, but when Arg41 is either ADP-ribosylated or mutated Arg128 becomes the preferred site of ADP-ribosylation (Ganesan et al. 1998). Arg41 and Arg128 are localized at the opposite sides of Ras molecule, indicating that ExoS could recognize multiple sites on Ras. While ExoS has a broad range of substrates relative to ExoT, it is not a nonspecific enzyme. In vitro and in vivo ExoS ADP-ribosylated a subset of proteins in mammalian cells. ExoS is a polysubstrate specific ADP-ribosyltransferase.

Polysubstrate specificity has been observed in several protein families, such as multidrug transporters, soluble multidrug recognizing proteins, and shark antibodies (Marchalonis et al. 1998). While the mechanism underlying polysubstrate specificity is not well understood, current biochemical and structural studies of multidrug transporters and their drug ligands indicate that polysubstrate specificity is due to a combination of hydrophobic effect, electrostatic attraction, and conformational complementarity, rather than a precise network established by hydrogen bonds and other specific interactions between proteins and ligands (Zheleznova et al. 1999, 2000; Neyfakh 2002). The hydrophobic interaction between proteins and ligands excludes interference of water molecules, which is in turn augmented by the electrostatic interaction surrounding the hydrophobic residues.

For the multidrug transporters, ligand property is not a strict factor in the binding mechanism, as molecules that contain hydrophobic and charged residues on the surface and physically fit into the binding site without including water molecules can be ligands. Different ligands interact with different subset of residues in the binding pocket. Compared to multidrug transporters, ExoS displays similar properties, requiring hydrophobic residues and charged residues to ADP-ribosylate substrates. ExoS may recognize multiple substrates through a similar mechanism to the multidrug transporters.

Evolutionary relationship and relative roles of ExoS and ExoT

Although bacterial ADP-ribosylating toxins target diverse mammalian proteins, structural and biochemical studies suggest that bacterial ADP-ribosyltransferases have similar 3-D structural backbones, which include a conserved NAD binding pocket and a conserved catalytic glutamate residue (Han et al. 1999, 2001). This indicates that the ADP-ribosylating enzymes evolved from a common ancestral ADP-ribosyltransferase, where the mechanisms of NAD binding and catalysis are relatively conserved, while the mechanism of substrate recognition diverged under various selection pressures. The phylogenetic trees derived from primary amino acid sequences of C3 exoenzyme, Vip2, Iota, C2 exoenzyme, ExoS, and ExoT show that ExoT is more closely related to the C2- or C3-like ADP-ribo-

sylating toxins than ExoS. ExoT and ExoS are probably the products of gene duplication, where the original gene product maintained protein function and the duplicated gene product diverged to attain a second independent function.

There are arguments for either ExoT or ExoS being the original gene product. Based upon prevalence among clinical isolates, *exoT* can be argued as the original gene, since *exoT* was more ubiquitous in strains of *P. aeruginosa* than *exoS.* Lomholt and Kilian et. al. screened clinical isolates of *P. aeruginosa* (145 strains) for the distribution of virulent factors (Lomholt et al. 2001); 59% of the 145 strains possess both *exoT* and *exoS*, 34% possess only *exoT*, and 6% possess only *exoS.* In another survey, 49% of *s*trains from patients with keratitis possessed only *exoT*, while 80%–100% strains from urine, lungs, wounds, and feces possessed a combination of *exoS* and *exoT*. Thirty-four percent of the 145 isolates contain *exoU*, another type-III cytotoxin recently characterized as a lipase (Sato et al. 2003). The presence of both *exoS* and *exoU* were essentially mutually exclusive. Based upon prevalence, *exoT* appears to be the more stable genotype and thus one can argue that *exoS* may have evolved and diverged from *exoT* to attain a novel property for polysubstrate recognition through gene duplication. In contrast, in environmental isolates ExoS is a common type-III cytotoxin, which could implicate ExoS as the progenitor gene product (Lanotte et al. 2004).

Biochemical studies can argue for the designation of either *exoT* or *exoS* as the progenitor. The biochemical studies that support the divergence of ExoS from ExoT through gene duplication include the determination that region A of ExoT is the Crk-specific recognition motif, while region A of ExoS does not play a role in substrate recognition. C3 and C3stau2 have slightly different substrate specificity, and show different molecular electrostatic surfaces on region A and E, indicating that region A of C3 ADP-ribosylating toxins also plays a role in substrate recognition. Therefore, upon duplication ExoS may have first become a nonfunctional duplicon that had limited affinity for Crk proteins due to mutations in region A and then evolved the polyspecificity of substrate recognition making ExoS a unique ADP-ribosylating enzyme relative to ExoT and the ADP-ribosylating enzymes from different species. This further indicates that ExoT and other orthologous ADP-ribosylating enzymes evolved at an earlier age, while ExoS evolved from ExoT paralogously through gene duplication. While paralogous proteins have dramatically different functions, they usually maintain similar 3-D structures. An important question is: can ExoS gain the polysubstrate specificity during evolution from ExoT without dramatic structural alterations? When only a limited number of residues of ExoT were replaced with residues from ExoS, the chimeras gained the ability to ADP-ribosylate different subsets of ExoS targets. These mutations do not appear to have dramatic structural alterations, since they have similar NAD glycohydrolase activity. Current studies from multi-drug transporters also indicate that the polyspecificity has arisen independently many times in protein evolution, without dramatic structural changes (Neyfakh 2002). Therefore, ExoS could gain the polysubstrate specificity during evolution from ExoT without dramatic structural alterations.

Biochemical properties that argue for the designation of ExoS as the progenitor and that exoT originated from gene duplication include the limited capacity for ExoT to catalyze the NAD glycohydrolase reaction, a common activity of other ADP-ribosylating proteins, where ExoT catalyzes the NAD glycohydrolase reaction at ~2% of the rate of ExoS. Second, ExoS and ExoT diverge in the primary amino acid sequence within the ARTT motifs, where ExoS has an aromatic residue that is conserved in most ADP-ribosylating

enzymes, while ExoT has a glutamate residue. Third, ExoS shows a limited capacity to ADP-ribosylate Crk, the preferred target of ExoT, while ExoT does not recognize the preferred targets of ExoS. Finally, a chimera that had the α-1 of ExoT engineered into ExoS efficiently ADP-ribosylated both ExoS targets and ExoT targets. This ExoS/ExoT chimera could represent an intermediate in the evolution of ExoS to ExoT, where α-1 evolved into an efficient Crk binding site and subsequently lost the ability to recognize ExoS targets through mutations within the ARTT motif (region C) and active site loop (region B).

Initially, since ExoT appeared to contain a "defective" ADP-ribosyltransferase domain relative to ExoS, the roles of ExoS and ExoT in bacterial pathogenesis and the selection for *P. aeruginosa* to maintain a "defective" gene, *exoT*, was unclear. The ADP-ribosylation of different eukaryotic proteins indicates that ExoS and ExoT play distinct roles in bacterial pathogenesis. While ExoS and ExoT are involved in antiphagocytosis, ExoT appears to be the major contributor for antiphagocytosis in most *P. aeruginosa* strains (Fleiszig et al. 1997; Cowell et al. 2000; Garrity-Ryan et al. 2000). While the RhoGAP domains of ExoS and ExoT stimulate reorganization of the actin cytoskeleton through inactivation of Rho, Rac, and Cdc42, which contributes to antiphagocytosis, this modulation is a reversible transient process. Moreover, the RhoGAP activity of ExoS and ExoT may be downregulated by ADP-ribosylation at their RhoGAP domains after delivery into host cells (Riese et al. 2002). Therefore, the ADP-ribosylation of Crk proteins by ExoT may represent the major contributor for antiphagocytosis. The ADP-ribosylation domain of ExoS is cytotoxic to mammalian cells. The mechanism of cell death caused by ExoS ADP-ribosyltransferase is not clear, but may be in part due to its polysubstrate specificity. The identification of new targets of ExoS ADP-ribosyltransferase will facilitate our understanding of ExoS-induced cell death. Recent studies show that the ADP-ribosylation activity of ExoS induces apoptosis of host cells (Frithz-Lindsten et al. 1997; Kaufman et al. 2000; Jia et al. 2003). Therefore, ExoS and ExoT are two type-III virulence factors that play distinct roles to disarm host defense, allowing the establishment of bacterial colonization.

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