**Pseudomonas aeruginosa** ExoS ADP-ribosyltransferase inhibits ERM phosphorylation

Anthony W. Maresso,1 Qing Deng,1 Michael S. Pereckas,2 Bassam T. Wakim2 and Joseph T. Barbieri1*

1Department of Microbiology and Molecular Genetics and 2Protein-Nucleic Acid Facility, Medical College of Wisconsin, Milwaukee, WI 53226, USA.

**Summary**

*Pseudomonas aeruginosa* causes life-threatening infections in compromised and cystic fibrosis patients. Pathogenesis stems from a number of virulence factors, including four type III translocated cytotoxins: ExoS, ExoT, ExoY and ExoU. ExoS is a bifunctional toxin: the N terminus (amino acids 96–219) encodes a Rho GTPase Activating Protein (GAP) domain. The C terminus (amino acids 234–453) encodes a 14-3-3-dependent ADP-ribosyltransferase domain which transfers ADP-ribose from NAD onto substrates such as the Ras GTPases and vimentin. Ezrin/radixin/moesin (ERM) proteins have recently been identified as high-affinity substrates for ADP-ribosylation by ExoS. Expression of ExoS in HeLa cells led to a loss of phosphorylation of ERM proteins that was dependent upon the expression of ADP-ribosyltransferase activity. MALDI-MS and site-directed mutagenesis studies determined that ExoS ADP-ribosylated moesin at three C-terminal arginines (Arg553, Arg560 and Arg563), which cluster Thr558, the site of phosphorylation by protein kinase C and Rho kinase. ADP-ribosylated-moesin was a poor target for phosphorylation by protein kinase C and Rho kinase, which showed that ADP-ribosylation directly inhibited ERM phosphorylation. Expression of dominant active-moesin inhibited cell rounding elicited by ExoS, indicating that moesin is a physiological target in cultured cells. This is the first demonstration that a bacterial toxin inhibits the phosphorylation of a mammalian protein through ADP-ribosylation. These data explain how the expression of the ADP-ribosylation of ExoS modifies the actin cytoskeleton and indicate that ExoS possesses redundant enzymatic activities to depolymerize the actin cytoskeleton.

**Introduction**

*Pseudomonas aeruginosa* is an opportunistic pathogen that is responsible for 10–20% of nosocomial infections (Bodey et al., 1983). *P. aeruginosa* virulence is multifactorial and includes four type III cytotoxins: ExoS, ExoT, ExoY and ExoU (Frank, 1997). Expression of the Type III cytotoxins is associated with poor clinical outcomes in patients with lung infections by *P. aeruginosa* (Hauser et al., 2002). ExoS is a bifunctional toxin consisting of an N-terminal Rho GTPase Activating Protein (Rho GAP) domain (residues 96–219) and a C-terminal ADP-ribosyltransferase domain (residues 234–453). The Rho GAP domain of ExoS stimulates the hydrolysis of the terminal phosphate of GTP on GTP-bound Rho GTPases, inactivating RhoA, Rac1 and Cdc42 (Goehring et al., 1999; Krall et al., 2002). Arg146 is a catalytic residue for the expression of Rho GAP activity (Goehring et al., 1999; Wurtele et al., 2001).

ADP-ribosyltransferase activities of ExoS and ExoT are involved in *P. aeruginosa* virulence (Garrity-Ryan et al., 2004). The 14-3-3-dependent ADP-ribosyltransferase domain of ExoS is poly-substrate specific and transfers ADP-ribose from NAD onto several substrates, including the small monomeric GTPases, vimentin (Coburn and Gill, 1991; Fu et al., 1993; Ganesan et al., 1998; 1999; McGuffie et al., 1998; Riese et al., 2001; Fraylick et al., 2002; Henriksson et al., 2002a,b; Maresso et al., 2004), and the anti-stress protein Hsp27 (A.W. Maresso and J.T. Barbieri, unpubl. data). Glu381 is an active site residue for the expression of ADP-ribosyltransferase activity (Liu et al., 1996). A Membrane Localization Domain (MLD) between amino acids 51–72 targets ExoS to a perinuclear/ER-like region in mammalian cells (Pederson et al., 2002; Krall et al., 2004). Deletion of the MLD allows for the purification of ExoS from *Escherichia coli* (Maresso and Barbieri, 2002).

Expression of ExoS in HeLa cells resulted in cell rounding that was independent of the expression of Rho GAP activity and due to expression of ADP-ribosylation activity (Maresso et al., 2004). During a probe to identify possible mechanisms responsible for the reorganization of the actin cytoskeleton by ADP-ribosylation, Ezrin, radixin and
moesin (ERM) were identified as high-affinity (Km ~0.4 \mu M) substrates of ExoS ADP-ribosyltransferase and observed to be ADP-ribosylated by ExoS in HeLa cells (Maresso et al., 2004). ERM proteins are adaptors between the actin cytoskeleton and receptor complexes (CD44 and EBP50) at the plasma membrane, which link signals transmitted from the plasma membrane to F-actin filaments as well as scaffolds for cytoskeletal structures such as microvilli. ERM proteins also facilitate the activation of Rho by exchanging Rho Guanine Disassociation Inhibitor (RhoGDI) for Dbl, a Rho guanine nucleotide exchange factor (GEF), which promotes the binding of GTP to Rho. ERM proteins are activated by phosphorylation of a C-terminal threonine residue (Thr558 in moesin). Phosphorylation of Thr558 relieves the intra-molecular association of the N terminus and C terminus of moesin, allowing both termini to interact with upstream or downstream effectors (Tsukita et al., 1994; Takahashi et al., 1997; 1998; Matsui et al., 1998; Pietromonaco et al., 1998; Reczek and Bretscher, 1998; Simons et al., 1998; Bretscher et al., 2000; Pearson et al., 2000). The current study investigates how ADP-ribosylation affects the ERM proteins.

**Results**

**Type III-delivered ExoS decreases ERM phosphorylation**

The activation state of the ERM proteins is regulated by the phosphorylation of Thr558 (Tsukita et al., 1994; Takahashi et al., 1997; 1998; Matsui et al., 1998; Pietromonaco et al., 1998; Reczek and Bretscher, 1998; Simons et al., 1998; Bretscher et al., 2000; Pearson et al., 2000). Initial experiments measured the effect of ADP-ribosylation on the phosphorylation state of moesin during infection. HeLa cells infected with *P. aeruginosa* PA103 expressing an ADP-ribosyltransferase active but GAP inactive form of ExoS (A’G+) showed an approximately threefold reduction in the phosphorylation state of ERM proteins as determined by α-P-Thr558 Western blot analysis compared with HeLa cells infected with *P. aeruginosa* PA103 transformed with the pUCP vector control (Fig. 1A). The reduced phosphorylation state of Thr558 was not due to changes in the steady state of ERM proteins (α-ERM) or varied protein in the samples (α-actin). Controls confirmed the presence of cell-associated ExoS in HeLa cells infected with *P. aeruginosa* PA103 expressing ExoS (A’G+) (α-ExoS). HeLa cell lysates subjected to 10% SDS-PAGE (to separate ezrin/radixin from moesin) showed that cells infected with *P. aeruginosa* expressing ExoS (A’G+) had reduced phosphorylation states for ezrin/radixin and moesin (Fig. 1B). Reduction in the steady state ERM phosphorylation increased with the time of infection, with a fivefold reduction observed at later time points during infection (Fig. 1C). Cell rounding, an indicator of cytoskeletal disruption and precursor to cell death elicited by ExoS ADP-ribosyltransferase, was detected at ~200 min post infection with ~70% of the cells rounded at 220 min (Fig. 1D). There was little cell rounding of HeLa cells infected with *P. aeruginosa* PA103 (pUCP vector control).

To determine if expression of ADP-ribosyltransferase activity of ExoS was required for the reduction in ERM phosphorylation, HeLa cells were infected with *P. aeruginosa* (PA103) expressing ExoS (A’G+), Rho GAP inactive ExoS (A’G+), ADP-ribosyltransferase inactive ExoS (A’G+), or both Rho GAP and ADP-ribosyltransferase inactive ExoS (A’G+) and ERM phosphorylation was measured. HeLa cells intoxicated with ExoS that possessed ADP-ribosyltransferase activity, ExoS (A’G+) or ExoS (A’G+), had lower steady state levels of ERM phosphorylation relative to HeLa cells intoxicated with ExoS that did not express ADP-ribosyltransferase activity, ExoS (A’G+) or ExoS (A’G+) (Fig. 1E). At the end of this experiment, the amount of HeLa cells rounded upon intoxication with ExoS (A’G+) or ExoS (A’G+) were similar (~80%), which indicated the process of cell rounding was not sufficient to reduce ERM phosphorylation.

**ExoS ADP-ribosylates multiple Args within the C-terminal Arg cluster of moesin**

Initial experiments showed that ExoS ADP-ribosylated recombinant moesin to ~0.3 mol ADP-ribose per mol moesin and that 45% of moesin migrated by SDS-PAGE with an apparent higher molecular weight than unmodified moesin, indicating that about half of the moesin was available for ADP-ribosylation (data not shown). ADP-ribosylated moesin was digested with trypsin and subjected to HPLC separation. After two rounds of HPLC a major radioactive fraction was isolated. MALDI-MS analysis of the major radioactive fraction identified three peptides that corresponded to ADP-ribosylated-tryptic peptides of moesin: two minor peptides, 558–570 (2141.78 Da, ~80%), which indicated the process of cell rounding was not sufficient to reduce ERM phosphorylation.
moesin (R553, R560, R563 and R570) were conserved within the ERM proteins. Based upon the localization of these Args within the poly-basic region of moesin and the MALDI-MS analysis of ADP-ribosylated moesin, R550, R553, R560, R563 and R570 were tested directly as potential sites of ADP-ribosylation. Each Arg was changed to Ala and the mutated proteins were subjected to a linear velocity ADP-ribosylation assay versus Wt-moesin. While ExoS ADP-ribosylated moesin(R550A), moesin(R570A) and Wt-moesin at similar rates, moesin(R553A), moesin(R560A) and moesin(R563A) were less efficient targets for ADP-ribosylation by ExoS (Fig. 2B). Together, the data showed that ExoS ADP-ribosylated moesin on multiple Args, including R553, R560 and R563.

ADP-ribosylation of moesin by ExoS inhibits phosphorylation by protein kinase C on Thr558

In cells, ADP-ribosylation could reduce the steady state levels of ERM phosphorylation by induction of ERM

| Peptide-mass (Da) | Corresponding residues of moesin | # of 541 Da (ADP-ribose) per peptide |
|------------------|---------------------------------|-------------------------------------|
| 1 (2141.78)      | 558–570                         | 1                                   |
| 2 (2404.97)      | 554–563                         | 2                                   |
| 3 (2399.93)      | 538–553                         | 1                                   |

Ten micrograms of moesin was ADP-ribosylated with ExoS(Δ51-72), subjected to tryptic digestion, and HPLC separation. Radioactive fractions were combined and subjected to a second round of HPLC analysis, using a shallower acetonitrile gradient. The major radioactive peak was subjected to MALDI-MS analysis. Identities were established from the predicted masses of tryptic peptides. Results are representative of one of three independent determinations.
dephosphorylation by a phosphatase or inhibition of ERM phosphorylation by a kinase. To distinguish between these two possibilities, in vitro ERM phosphorylation was measured using protein kinase C (PKC) and Rho kinase (ROK). While the physiological kinase for ERM phosphorylation is currently in question (Yokoyama et al., 2005), PKC and ROK phosphorylate moesin on Thr558 (Matsui et al., 1998; Pietromonaco et al., 1998), which provides an in vitro assay to measure the effect of ADP-ribosylation on ERM phosphorylation. To enhance the resolution of the analysis, a C-terminal peptide of moesin, moesin(404–577), was used as the substrate, because full-length moesin is a poor substrate for in vitro phosphorylation by PKC and ROK (Matsui et al., 1998). Moesin(404–577) is considered to be structurally and functionally representative of the active C terminus of moesin (Gary and Bretscher, 1995; Huang et al., 1999; Pearson et al., 2000). ExoS ADP-ribosylated moesin(404–577) about three times more efficiently than full-length moesin on a molar basis, consistent with the C-terminal Arg being more accessible in moesin(404–577) (Fig. 3A). Taken together, moesin(404–577) appeared to be an efficient substrate of ADP-ribosylation by ExoS and was used in subsequent experiment to determine the effect of ADP-ribosylation on the ability of kinases to phosphorylate moesin.

The relative efficiency of the phosphorylation of ADP-ribosylated moesin(404–577) by PKC was reduced approximately 10-fold during the linear phase of the kinase reaction (0–60 min) and approximately 15-fold at saturation compared with unmodified moesin(404–577) (Fig. 3B). Moesin(T558D) was not phosphorylated in this reaction, indicating the site of phosphorylation being assayed was Thr558 (data not shown). Other experiments showed that the relative efficiency of the phosphorylation of ADP-ribosylated-moesin(404–577) by ROK was reduced approximately threefold compared with phosphorylation of unmodified moesin(404–577) (data not shown). Collectively, the data show that ADP-ribosylation directly inhibits the phosphorylation of Thr558 of moesin by host kinases and provides a mechanism for the reduced steady state level of ERM phosphorylation observed during ExoS intoxication.

Dominant active moesin interferes with actin cytoskeleton reorganization elicited by ExoS ADP-ribosyltransferase activity

Regulation of the actin cytoskeleton is linked to the activation state of moesin, which prompted the measurement of the ability of dominant active-moesin [DA-moesin; Moesin(T558D)] to inhibit cell rounding by ExoS. HeLa cells were transfected overnight with plasmids encoding GFP, GFP-moesin or GFP-DA-moesin. While GFP-
moesin was localized to the cytosol and membranes, GFP-DA-moesin was primarily localized to the cell membrane (Fig. 4A). Expression of DA-moesin inhibited the cell rounding by type III-delivered ExoS (G–A+), while expression of either GFP or GFP-moesin did not interfere with cell rounding by ExoS (Fig. 4B).

P. aeruginosa expressing a vector control did not stimulate cell rounding in normal or moesin-transfected cells. Time-course experiments observed that the inhibitory effect of DA-moesin on ExoS (G–A+) -mediated cell rounding was most apparent at early times of infection. The ability of DA-moesin to inhibit the action of the ExoS supported a role for moesin as a physiological target for the ADP-ribosyltransferase action of ExoS. Under similar conditions, DA-Radixin did not protect HeLa cells from rounding elicited by ExoS (data not shown). The overall subcellular distribution of moesin or radixin did not change during ExoS intoxication, but the majority of ADP-ribosylated moesin and ADP-ribosylated-radixin was membrane associated. The significance of this localization is currently under investigation.

Discussion

MALDI-MS analysis showed that ExoS ADP-ribosylated moesin at multiple ArgS, including R553, R560 and R563. Multiple sites of ADP-ribosylation on Ras by ExoS has also be observed, where Arg41 appeared to be the primary ADP-ribosylation site and Arg123 was a secondary site of ADP-ribosylation (Ganesan et al., 1998). The structure of the C terminus of moesin (Fig. 2C) includes a poly-basic cluster of amino acids where R553, R560 and R563 are within 15 Å of each other. The inability to observe the ADP-ribosylation of R550, which is also within the poly-basic cluster, is consistent with guanidino group side-chain of R550 being positioned on the opposite side.

moesin was localized to the cytosol and membranes, GFP-DA-moesin was primarily localized to the cell membrane (Fig. 4A). Expression of DA-moesin inhibited the cell rounding by type III-delivered ExoS (G–A+), while expression of either GFP or GFP-moesin did not interfere with cell rounding by ExoS (Fig. 4B). P. aeruginosa expressing a vector control did not stimulate cell rounding in normal or moesin-transfected cells. Time-course experiments observed that the inhibitory effect of DA-moesin on ExoS (G–A+) -mediated cell rounding was most apparent at early times of infection. The ability of DA-moesin to inhibit the action of the ExoS supported a role for moesin as a physiological target for the ADP-ribosyltransferase action of ExoS. Under similar conditions, DA-Radixin did not protect HeLa cells from rounding elicited by ExoS (data not shown). The overall subcellular distribution of moesin or radixin did not change during ExoS intoxication, but the majority of ADP-ribosylated moesin and ADP-ribosylated-radixin was membrane associated. The significance of this localization is currently under investigation.

Discussion

MALDI-MS analysis showed that ExoS ADP-ribosylated moesin at multiple ArgS, including R553, R560 and R563. Multiple sites of ADP-ribosylation on Ras by ExoS has also be observed, where Arg41 appeared to be the primary ADP-ribosylation site and Arg123 was a secondary site of ADP-ribosylation (Ganesan et al., 1998). The structure of the C terminus of moesin (Fig. 2C) includes a poly-basic cluster of amino acids where R553, R560 and R563 are within 15 Å of each other. The inability to observe the ADP-ribosylation of R550, which is also within the poly-basic cluster, is consistent with guanidino group side-chain of R550 being positioned on the opposite side.

moesin was localized to the cytosol and membranes, GFP-DA-moesin was primarily localized to the cell membrane (Fig. 4A). Expression of DA-moesin inhibited the cell rounding by type III-delivered ExoS (G–A+), while expression of either GFP or GFP-moesin did not interfere with cell rounding by ExoS (Fig. 4B). P. aeruginosa expressing a vector control did not stimulate cell rounding in normal or moesin-transfected cells. Time-course experiments observed that the inhibitory effect of DA-moesin on ExoS (G–A+) -mediated cell rounding was most apparent at early times of infection. The ability of DA-moesin to inhibit the action of the ExoS supported a role for moesin as a physiological target for the ADP-ribosyltransferase action of ExoS. Under similar conditions, DA-Radixin did not protect HeLa cells from rounding elicited by ExoS (data not shown). The overall subcellular distribution of moesin or radixin did not change during ExoS intoxication, but the majority of ADP-ribosylated moesin and ADP-ribosylated-radixin was membrane associated. The significance of this localization is currently under investigation.

Discussion

MALDI-MS analysis showed that ExoS ADP-ribosylated moesin at multiple ArgS, including R553, R560 and R563. Multiple sites of ADP-ribosylation on Ras by ExoS has also be observed, where Arg41 appeared to be the primary ADP-ribosylation site and Arg123 was a secondary site of ADP-ribosylation (Ganesan et al., 1998). The structure of the C terminus of moesin (Fig. 2C) includes a poly-basic cluster of amino acids where R553, R560 and R563 are within 15 Å of each other. The inability to observe the ADP-ribosylation of R550, which is also within the poly-basic cluster, is consistent with guanidino group side-chain of R550 being positioned on the opposite side.

moesin was localized to the cytosol and membranes, GFP-DA-moesin was primarily localized to the cell membrane (Fig. 4A). Expression of DA-moesin inhibited the cell rounding by type III-delivered ExoS (G–A+), while expression of either GFP or GFP-moesin did not interfere with cell rounding by ExoS (Fig. 4B). P. aeruginosa expressing a vector control did not stimulate cell rounding in normal or moesin-transfected cells. Time-course experiments observed that the inhibitory effect of DA-moesin on ExoS (G–A+) -mediated cell rounding was most apparent at early times of infection. The ability of DA-moesin to inhibit the action of the ExoS supported a role for moesin as a physiological target for the ADP-ribosyltransferase action of ExoS. Under similar conditions, DA-Radixin did not protect HeLa cells from rounding elicited by ExoS (data not shown). The overall subcellular distribution of moesin or radixin did not change during ExoS intoxication, but the majority of ADP-ribosylated moesin and ADP-ribosylated-radixin was membrane associated. The significance of this localization is currently under investigation.

Discussion

MALDI-MS analysis showed that ExoS ADP-ribosylated moesin at multiple ArgS, including R553, R560 and R563. Multiple sites of ADP-ribosylation on Ras by ExoS has also be observed, where Arg41 appeared to be the primary ADP-ribosylation site and Arg123 was a secondary site of ADP-ribosylation (Ganesan et al., 1998). The structure of the C terminus of moesin (Fig. 2C) includes a poly-basic cluster of amino acids where R553, R560 and R563 are within 15 Å of each other. The inability to observe the ADP-ribosylation of R550, which is also within the poly-basic cluster, is consistent with guanidino group side-chain of R550 being positioned on the opposite side.

moesin was localized to the cytosol and membranes, GFP-DA-moesin was primarily localized to the cell membrane (Fig. 4A). Expression of DA-moesin inhibited the cell rounding by type III-delivered ExoS (G–A+), while expression of either GFP or GFP-moesin did not interfere with cell rounding by ExoS (Fig. 4B). P. aeruginosa expressing a vector control did not stimulate cell rounding in normal or moesin-transfected cells. Time-course experiments observed that the inhibitory effect of DA-moesin on ExoS (G–A+) -mediated cell rounding was most apparent at early times of infection. The ability of DA-moesin to inhibit the action of the ExoS supported a role for moesin as a physiological target for the ADP-ribosyltransferase action of ExoS. Under similar conditions, DA-Radixin did not protect HeLa cells from rounding elicited by ExoS (data not shown). The overall subcellular distribution of moesin or radixin did not change during ExoS intoxication, but the majority of ADP-ribosylated moesin and ADP-ribosylated-radixin was membrane associated. The significance of this localization is currently under investigation.

Discussion

MALDI-MS analysis showed that ExoS ADP-ribosylated moesin at multiple ArgS, including R553, R560 and R563. Multiple sites of ADP-ribosylation on Ras by ExoS has also be observed, where Arg41 appeared to be the primary ADP-ribosylation site and Arg123 was a secondary site of ADP-ribosylation (Ganesan et al., 1998). The structure of the C terminus of moesin (Fig. 2C) includes a poly-basic cluster of amino acids where R553, R560 and R563 are within 15 Å of each other. The inability to observe the ADP-ribosylation of R550, which is also within the poly-basic cluster, is consistent with guanidino group side-chain of R550 being positioned on the opposite side.
of the cluster relative to the side-chains of R553, R560 and R563. R570 lies on the same face as Arg553, Arg560 and Arg563, but is farther away from the centre of the basic cluster, which may explain why Arg570 appears to be a minor site for ADP-ribosylation. The observation that ExoS ADP-ribosylated moesin(R553), moesin(R560) and moesin(R563) at slower rates than WT-moesin is consistent with each Arg being a target for ADP-ribosylation.

The reduced steady state phosphorylation of the ERM proteins in cultured cells appears to be due to the inability of protein kinases to phosphorylate ADP-ribosylated-moesin. The level of inhibition was more pronounced for PKC, most likely attributable to PKC’s approximately five-fold higher specific activity towards moesin than ROK.

Considering that the –OH side-chain of Thr558 is located on the same plane as Arg553, Arg560 and Arg563, steric hindrance by one or more ADP-ribose residue(s) may prevent either kinase from transferring a phosphate onto Thr558 (Fig. 5). This inhibition of the phosphorylation of ERM proteins by ADP-ribosylation may constitute a bipartite inhibitory mechanism by down-modulating two functions of the ERM proteins, Rho activation and actin polymerization. This mechanism of inhibition is unique among bacterial protein toxins and fits the paradigm of bacterial toxins targeting steps that are important for signal transduction. The inactivation of moesin indicates that ExoS is redundant for the inactivation of Rho functions, coupling to the Rho GAP activity within the N terminus of the toxin with the observed ADP-ribosylation of moesin. Rho GAP activity is non-covalent and reversible, while ADP-ribosylation is generally considered irreversible; perhaps ExoS evolved both activities to affect Rho for temporal down-modulation of actin dynamics. This parallels Yersinia pathogenesis where YopE (a Rho GAP) and YopT (a protease) both target the Rho GTPases, by non-covalent and covalent mechanisms respectively (Cornelis, 2000; Shao et al., 2002).

There are several bacterial toxins that modulate the phosphorylation status of mammalian proteins. Cytotoxic necrotizing factor 1 from E. coli constitutively activates the Rho GTPases Rho A, Rac 1 and Cdc42 to stimulate myosin light-chain phosphorylation in a ROK-dependent manner (Essler et al., 2003), while Pasteurella multocida toxin stimulates phosphorylation of Gαq at Tyr349 (Baldwin et al., 2003). Both toxins may also induce focal adhesion kinase Tyr397 phosphorylation, a modification that may play a role in the formation of complexes with Src proteins (Thomas et al., 2001). Further, p38 mitogen-activated protein kinase is phosphorylated after challenge with pneumolysin from Streptococcus pneumoniae (Stringaris et al., 2002). The mechanism for the induction of phosphorylation is not known. YopH dephosphorylates focal adhesion proteins, including p130Cas to inhibit phagocytosis (Bliska et al., 1991; Black and Bliska, 1997). CagA has been reported to target ERM protein function. Upon translocation through the type-IV secretion system, CagA induces actin rearrangements and dephosphorylation of ezrin in a Src inactivation-dependent manner, but undefined mechanism (Selbach et al., 2004).

While poly-substrate specificity complicates the ability to determine the physiological significance of host proteins that are ADP-ribosylated by ExoS, the ability of DA-moesin to inhibit cell rounding elicited by ExoS supports a role for moesin as a physiological target. The inhibition of phosphorylation of ERM proteins through ADP-ribosylation is a novel activity for a bacterial toxin and adds to the diversity of modulator functions that bacterial toxins exert on mammalian signalling pathways.

**Experimental procedures**

**Plasmids and reagents**

Construction of pUCP-ExoS, pUCP-ExoS E381D, pUCP-ExoS R146K and pUCP-ExoS R146K E381D have been described (Vallas et al., 1999; Riese et al., 2002). Recombinant forms of ExoS were expressed in P. aeruginosa PA103 (ΔexoU, exoT::Tc). This allowed expression of a single type III effector, as PA103 (ΔexoU, exoT::Tc) lacks the genes encoding ExoS and ExoY and has been engineered with a deletion in the gene encoding ExoU.
and a Tc-encoding transposon inserted into the gene encoding ExoT. The construction of pET-(His)_6-moesin has been described (Maresso et al., 2004). Moesin(404–577) was generated as follows: pET-(His)_6-moesin was used as a template to amplify DNA encoding amino acids 404–577 using primers forward 5'-GATCGATCTCCGAGCATATGCGTCCCGGACCA GAAAAAG-3' and reverse 5'-GATCGATCGAATTTAGTGCAG CGGCATAGCTCAATTGGCTACG-3' with unique XhoI and EcoRI (underlined) restriction sites respectively. PCR products were digested with XhoI and EcoRI and ligated into pET15b to create pET15b-(His)_6-Moesin(404–577). Moesin mutations R550A, R553A, R560A, R570A and T558D were generated using QuikChange® site-directed mutagenesis (Stratagene, Cedar Creek, TX) according to manufacturer's instructions. α-Phospho-ERM rabbit antibody was from Cell Signaling Technology (Beverly, MA). α-HA mouse and α-actin rabbit antibodies were from Covance (Princeton, NJ). SuperSignal (PicoWest) Chemiluminescence was from Pierce Biotechnology (Rockford, IL).

Protein expression and purification

Recombinant proteins were purified as previously described for ExoS(Δ51-72) and for 14-3-3 (Masters et al., 1999; Maresso and Barbieri, 2002). Identification of the site of ADP-ribosylation on moesin. Recombinant moesin was ADP-ribosylated to saturation as follows: ExoS(Δ51-72) (4.5 nM) were incubated in 100 μl reactions for 5 h in the presence of 14-3-3 (250 nM), 10 μM [32P]NAD (0.2 μCi), moesin (10 μg) and 50 mM Tris-HCl (pH 7.6), containing 2.0 μg of bovine serum albumin. The reactions were subjected to SDS-PAGE and radiolabelled moesin were subjected to scintillation spectrometry. Peptide analysis: acetone precipitants (90% final) were digested with 1 μg of trypsin for 12 h at 37°C in 100 μl of 50 mM Tris-HCl(pH 7.6). Tryptic peptides were applied in 5 μl acetonitrile, 6 M guanidine to a C18 100×2.1 mm Brownlee Spheri-5RP/18 (5 μM pore size) column for HPLC analysis (gradient 5–65% acetonitrile, 40 min run time). A second HPLC analysis of radiolabelled fractions was performed to further resolve peaks (gradient 5–50% acetonitrile, 60 min run time). HPLC purified tryptic peptides were lyophilized and dissolved in 15 μl of 0.1% trifluoroacetic acid (TFA). C18 Zip Tips (Millipore, Bedford, MA) were equilibrated successively in 15 μl of 100% acetonitrile, 15 μl of 50% acetonitrile (H₂O), and 15 μl of 0.1% TFA (H₂O). Peptides were bound to the resin, washed twice with 0.1% TFA in H₂O, eluted with 2 μl of 60% acetonitrile in 0.1% TFA (H₂O saturated with α-cyano-4-hydroxycinnamic acid). C18-purified tryptic peptides ionized by a N₂ UV laser using a PE-pro mass spectrometer (Applied Biosystems, Foster City, CA). Two hundred laser shots were conducted at an accelerating voltage of 25 000 V and laser intensity of 2075 (repetition rate 3 Hz).

Enzyme activity

Linear velocity determination. Reactions (20 μl) contained: ExoS(ΔMLD) (4.5 nM), FAS (250 nM), 10 μM [32P]NAD (0.2 μCi), and moesin variants (500 nM) in 50 mM Tris-HCl(pH 7.6). Reactions were incubated for 10 min (mutated moesin proteins) or 0–180 min [moesin(404–577)] and subjected to SDS-PAGE.

Stoichiometric determination. Moesin stoichiometric analysis proceeded as described for linear velocity determination except that reactions were run for 5 h (determined to be saturating for ADP-ribosylated). Reactions were subjected to SDS-PAGE, gels were dried, and the shift in apparent molecular weight due to ADP-ribosylation was measured by densitometry or radiolabelled moesin bands were assayed by scintillation counting.

Phosphorylation of moesin in cultured cells

HeLa cells (70% confluent, 6 well plate) were infected (m.o.i 8:1, bacteria/HeLa cell) with PA103 (ΔexoU, exoT::Tc) expressing pUCP-ExoS, pUCP-ExoS-R146K, pUCP-ExoS-E381D, pUCP-ExoS-R146K, E381D or pUCP for 160–220 min. At the indicated time, cells were lysed in 100 μl of SDS-PAGE sample buffer and subjected to SDS-PAGE and Western blot analysis to detect phosphorylated Moesin/ERM (1:2000), total ERM protein (1:2000), ExoS (HA-epitope 1:2000), and actin (1:10 000) (primary antibody dilution) and appropriate HRP-secondary antibody. Membranes were developed with ECL and exposed to X-ray film. In several experiments, cells were transfected overnight (Lipofectamine Plus, Invitrogen) with 300 ng of the indicated plasmid prior to infection by P. aeruginosa.
Bodey, G.P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) 
Coburn, J., and Gill, D.M. (1991) ADP-ribosylation of p21ras
Ganesan, A.K., Vincent, T.S., Olson, J.C., and Barbieri, J.T. 
Henriksson, M.L., Francis, M.S., Palmer, R., et al. (2002a) A nonphosphorylated 
14-3-3 binding motif on exoenzyme S that is functional in vivo. Eur J Biochem 269: 4921–4929. 
Henriksson, M.L., Sundin, C., Jansson, A.L., Forsberg, A., 
Palmer, R.H., and Hallberg, B. (2002b) Exoenzyme S 
show selective ADP-ribosylation and GAP activities 
towards small GTPases in vivo. Biochem J 367 (Pt3): 
617–628.
Huang, L., Wong, T.Y., Lin, R.C., and Furthmayr, H. (1999) 
Replacement of threonine 558, a critical site of phospho-
rylation of moesin in vivo, with aspartate activates F-actin 
binding of moesin. Regulation by conformational change. J 
Biol Chem 274: 12803–12810.
Knight, D.A., and Barbieri, J.T. (1997) Ecto-ADP- 
ribosyltransferase activity of Pseudomonas aeruginosa 
exoenzyme S. Infect Immun 65: 3304–3309.
Krall, R., Sun, J., Pederson, K.J., and Barbieri, J.T. (2002) In 
vivo rho GTPase-activating protein activity of Pseudomo-
as aeruginosa cytotoxin ExoS. Infect Immun 70: 360–367.
Krall, R., Zhang, Y., and Barbieri, J.T. (2004) Intracellular 
membrane localization of pseudomonas ExoS and Yersinia 
YopE in mammalian cells. J Biol Chem 279: 2747–2753.
Liu, S., Kulich, S.M., and Barbieri, J.T. (1996) Identification of 
glutamic acid 381 as a candidate active site residue of 
Pseudomonas aeruginosa exoenzyme S. Biochemistry 35: 
2754–2758.
McCuffie, E.M., Frank, D.W., Vincent, T.S., and Olson, J.C. 
(1998) Modification of Ras in euukaryotic cells by 
Pseudomonas aeruginosa exoenzyme S. Infect Immun 66: 
2607–2613.
Maresso, A.W., and Barbieri, J.T. (2002) Expression and 
purification of two recombinant forms of the type-III cyto-
toxin, Pseudomonas aeruginosa ExoS. Protein Expr Purif 
26: 432–437.
Maresso, A.W., Baldwin, M.R., and Barbieri, J.T. (2004) 
Ezrin/radixin/moesin proteins are high affinity targets for 
ADP-ribosylation by Pseudomonas aeruginosa ExoS. J 
Biol Chem 279: 38402–38408.
Masters, S.C., Pederson, K.J., Zhang, L., Barbieri, J.T., 
and Fu, H. (1999) Interaction of 14-3-3 with a nonphosphory-
lated protein ligand, exoenzyme S of Pseudomonas 
aeruginosa. Biochemistry 38: 5216–5221.
Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., 
Kabuchi, K., and Tsukita, S. (1998) Rho-kinase phospho-
rylates COOH-terminal threonines of ezrin/radixin/moesin 
(ERM) proteins and regulates their head-to-tail association. 
J Cell Biol 140: 647–657.
Pearson, M.A., Reczek, D., Bretscher, A., and Karplus, P.A. 
(2000) Structure of the ERM protein moesin reveals the 
FERM domain fold masked by an extended actin binding 
tail domain. Cell 101: 259–270.
Pederson, K.J., Krall, R., Riese, M.J., and Barbieri, J.T. 
(2002) Intracellular localization modulates targeting of 
ExoS, a type III cytotoxin, to eukaryotic signalling proteins. 
Mol Microbiol 46: 1381–1390.
Pietromonaco, S.F., Simons, P.C., Altman, A., and Elias, L. 
(1998) Protein kinase C-theta phosphorylation of moesin in 
the actin-binding sequence. J Biol Chem 273: 7594–7603.
Reczek, D., and Bretscher, A. (1998) The carboxyl-terminal 
region of EBP50 binds to a site in the amino-terminal 
domain of ezrin that is masked in the dormant molecule. J 
Biol Chem 273: 18452–18458.
ExoS ADP-ribosylation inhibits ERM phosphorylation

© 2006 The Authors
Journal compilation © 2006 Blackwell Publishing Ltd, Cellular Microbiology, 9, 97–105