Phosphorylation-independent interaction between 14-3-3 and exoenzyme S: from structure to pathogenesis

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14-3-3 proteins are phosphoserine/phosphothreonine-recognizing adapter proteins that regulate the activity of a vast array of targets. There are also examples of 14-3-3 proteins binding their targets via unphosphorylated motifs. Here we present a structural and biological investigation of the phosphorylation-independent interaction between 14-3-3 and exoenzyme S (ExoS), an ADP-ribosyl-transferase toxin of *Pseudomonas aeruginosa*. ExoS binds to 14-3-3 in a novel binding mode mostly relying on hydrophobic contacts. The 1.5 Å crystal structure is supported by cytotoxicity analysis, which reveals that substitution of the corresponding hydrophobic residues significantly weakens the ability of ExoS to modify the endogenous targets RAS/RAP1 and to induce cell death. Furthermore, mutation of key residues within the ExoS binding site for 14-3-3 impairs virulence in a mouse pneumonia model. In conclusion, we show that ExoS binds 14-3-3 in a novel reversed orientation that is primarily dependent on hydrophobic residues. This interaction is phosphorylation independent and is required for the function of ExoS.

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Introduction

14-3-3 proteins are a group of abundant conserved intracellular molecules produced in plants, vertebrates and higher eukaryotes with no intrinsic enzymatic activity (Aitken, 2006). The human genome contains several distinct 14-3-3 genes, denoted β , γ , ϵ , η , σ , τ and ζ , which function as homo- or heterodimers (Ichimura *et al*, 1988; Toker *et al*, 1992; Chaudhri *et al*, 2003; Aitken, 2006). 14-3-3 proteins are promiscuous and interact with more than 200 partners with a broad range of functions. In plants, 14-3-3 binding partners are involved in biosynthetic metabolism, transport of ions and regulation of growth (Moorhead *et al*, 1999; Roberts, 2003; Wurtele *et al*, 2003). In humans, these partners play a role in physiological processes such as cell proliferation, cell cycle progression, protein trafficking, rearrangement of the cytoskeleton, apoptosis and survival (Mackintosh, 2004).

In the majority of cases, 14-3-3 proteins recognize phosphorylated peptides in their binding partners. Crystal structure analyses of the apo 14-3-3 dimer alone, first reported for human 14-3-3 τ and 14-3-3 ζ (Liu *et al*, 1995; Xiao *et al*, 1995), and of 14-3-3 in complex with peptides or native binding partners have revealed a basic cluster in the amphipathic groove of each monomer that mediates the interaction of 14-3-3 with the phospho-amino-acid residues of its interaction partners (Yaffe *et al*, 1997; Rittinger *et al*, 1999). Each 14-3-3 dimer contains two binding pockets and can interact with canonical binding motifs, defined as mode I (RSXPSP) and mode II (RX Φ XpSP) (where Φ is an aromatic or aliphatic residue and X is any type of residue) (Yaffe *et al*, 1997). Recently, the structural basis for the activation of the plant plasma membrane H⁺-ATPase by the fungal toxin fusicoccin was uncovered (Svennelid *et al*, 1999; Wurtele *et al*, 2003). The crystal structure showed how fusicoccin stabilizes the complex between 14-3-3 and the C-terminus of the H⁺-ATPase thereby relieving the autoinhibitory activity of this regulatory domain (Wurtele *et al*, 2003). Based on the homology between the C-terminal 14-3-3 binding motif of the serotonin-N-acetyltransferase (AANAT, arylalkylamine-N-acetyltransferase) and H⁺-ATPase proteins, a so-called mode III binding motif has been proposed (Ganguly *et al*, 2005). In addition, interactions between 14-3-3 proteins and its target partner(s) can also occur outside the amphipathic groove, which probably contributes to a stable three-dimensional configuration and provides an opportunity for conformational modulation of the target (Rittinger *et al*, 1999; Obsil *et al*, 2001; Coblitz *et al*, 2005, 2006).

14-3-3 also interacts in a phosphorylation-independent manner with some target proteins. These include human telomerase (hTERT) (Seimiya *et al*, 2000), the enteropathogenic *Escherichia coli* Tir protein (Patel *et al*, 2006), the amyloid β -protein precursor intracellular domain fragment (Sumioka *et al*, 2005) and the Exoenzyme S (ExoS) cytotoxin,

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secreted by the Gram-negative bacterium *Pseudomonas aeruginosa* (Masters *et al*, 1999; Henriksson *et al*, 2000b). ExoS has been the subject of particular interest. This toxin is secreted by the type III secretion system of *P. aeruginosa* (Yahr *et al*, 1996) and contributes to the infection this opportunistic pathogen causes in immunocompromised individuals, including those with cystic fibrosis, cancer, burn wounds and respiratory compromise requiring mechanical ventilation (Stryjewski and Sexton, 2003). This bifunctional toxin possesses an N-terminal Rho GTPase activating protein (GAP) activity (Goehring *et al*, 1999; Wurtele *et al*, 2001; Henriksson *et al*, 2002a) and a highly promiscuous C-terminally encoded ADP-ribosylation activity towards small GTP-binding proteins, such as the RAS family members (Coburn *et al*, 1989; Coburn and Gill, 1991; Henriksson *et al*, 2002b). Activation of the ADP-ribosyltransferase activity of ExoS results in cell death (Liu *et al*, 1996; Frithz-Lindsten *et al*, 1997) and, in animal models of pneumonia, increased bacterial persistence in the lung and decreased host survival (Shaver and Hauser, 2004).

The intracellular function of ExoS depends upon an interaction with 14-3-3 proteins (Fu *et al*, 1993). A phosphorylation-independent interaction with this cofactor is required for the ADP-ribosylation activity of ExoS (Masters *et al*, 1999; Henriksson *et al*, 2000b). Upon translocation of ExoS into the host cell, many small GTPases are modified, such as Ras and Rap1. This modification of Ras has been correlated with inhibition of Ras and Rap1 activation in cell culture systems (Henriksson *et al*, 2000a, b). Moreover, ExoS-mediated ADP-ribosylation is also evident in cells expressing a constitutively active variant of Ras but in this case inhibition of function does not occur. In these cells, induction of apoptosis by activation of caspase-3 is not observed, and ExoS-mediated cell death does not occur (Jansson *et al*, 2006). These observations suggest that the Ras GTPase is the dominant target by which ExoS mediates cell death (Jansson *et al*, 2006).

Although 14-3-3 clearly binds ExoS in the absence of phosphorylation, the nature of the structural interaction between these two proteins is unclear. It has been postulated that acidic aspartic residues within the 14-3-3 binding domain of ExoS(⁴²³LDSL⁴²⁸) might substitute for phosphorylated amino acids. In support of this idea, a synthetic peptide from a phage display library, with the sequence WLDLE, bound the amphipathic groove of 14-3-3 in a similar manner reminiscent of a phosphorylated mode-I peptide of pS-RAF-1-259 (Petosa *et al*, 1998; Masters *et al*, 1999). However, predictions of phosphorylation-independent interactions mediated by acidic residues (phospho-mimicking) have recently been questioned. This is because an ExoS variant harbouring mutations in aspartic acid at positions 424 and 427 within the putative binding site for 14-3-3 of ExoS(⁴²³LDSL⁴²⁸) was as aggressive as wild-type ExoS in its ability to induce cell death and modify endogenous ExoS targets within the cell (Yasmin *et al*, 2006).

Here we have further elucidated the interaction between 14-3-3 and ExoS using crystal structural analysis in combination with biochemical and biological assays. Our structural analysis shows that leucine residues at positions 422, 423, 426 and 428 of ExoS are essential for binding to 14-3-3. They interact via an extensive hydrophobic surface within the 'roof' of the amphipathic groove of 14-3-3. Several electrostatic interactions involving His418, Asp424 and Asp427 of

ExoS complete this interaction. The importance of these molecular contacts was demonstrated by a functional biochemical analysis of ExoS variants employing bacterial infection assays in mammalian cell lines and by virulence assays using a mouse pneumonia model system. Our results show that the phosphorylation-independent interaction between ExoS and 14-3-3 occurs in a completely novel reversed fashion and is dependent on hydrophobic interactions rather than being electrostatically driven, as occurs with mode I-III consensus peptides.

Results

The 14-3-3ζ/ExoS complex

14-3-3 and ExoS interact in a phosphorylation-independent manner, but the precise mechanism has not been characterized (Fu *et al*, 1993; Yasmin *et al*, 2006). We therefore undertook a structural analysis of the interaction between these two proteins. To obtain crystals of the 14-3-3ζ and ExoS complex suitable for X-ray diffraction, a polyclonally produced and purified 14-3-3/ExoS complex was mildly trypsinized before setting up crystallization trials. This procedure yielded crystals that diffracted to 1.5 Å, PDB code 2002. The 14-3-3ζ/ExoS (416–430) structure was solved by molecular replacement using phases from the 14-3-3ζ apo structure. For the 14-3-3 dimer, we found interpretable density for 226 (14-3-3ζ A) and 232 (14-3-3ζ B) out of 246 residues. For the ExoS peptide, the density allowed building of 15 residues (⁴¹⁶SGHGQGLLDALDLAS⁴³⁰) complexed to one 14-3-3ζ A monomer and 10 residues (⁴²¹GLLDALDLAS⁴³⁰) complexed to the second monomer, 14-3-3ζ B. The root mean square deviation of the backbone Cα-atoms is 0.56 Å comparing 232 residues of one 14-3-3ζ monomer with the corresponding 14-3-3ζ monomer previously solved by Rittinger *et al* (1999) (PDB code 1QJB), which is in the range of the experimental coordination error and indicating the conserved 14-3-3 dimer structure. Data statistics are summarized in Table I. The 14-3-3 dimer displays the characteristic W-like shape with each monomer constituting an amphipathic groove that harbours the ExoS peptide

Table I Crystallographic data collection and refinement statistics

Cell dimensions	$a = 71.33 \text{ Å}$, $b = 72.24 \text{ Å}$, $c = 125.66 \text{ Å}$
Space group	P2 ₁ 2 ₁ 2 ₁
Resolution (Å)	20–1.5 (1.6–1.5)
Measured reflections	962 939 (126 882)
Unique reflections	103 943 (17 926)
Completeness (%)	99.5 (98.5)
I/σ	16.2 (3.6)
R_{sym} (%) ^a	7.4 (56.0)
R_{cryst} (%) ^b	15.1
R_{free} (%) ^c	21.1
Protein atoms	4882
Ligand atoms	18
Solvent atoms	721

Data for the outermost shell are shown in parentheses. RMSD, root mean square deviations from ideal geometry.

^a $R_{\text{sym}} = \sum |I_{hi} - \langle I_{hi} \rangle| / \sum I_{hi}$, where I_{hi} is the scaled observed intensity of the i th symmetry-related observation of the reflection h and $\langle I_{hi} \rangle$ the mean value.

^b $R_{\text{cryst}} = \sum h |F_{oh} - F_{ch}| / \sum h F_{oh}$, where F_{oh} and F_{ch} are the observed and calculated structure factor amplitudes for reflection h .

^c R_{free} was calculated as R_{cryst} with 5% of the data omitted from structure refinement.

Figure 1 Structure of 14-3-3 ζ complexed with ExoS peptides. (A) Structure of the 14-3-3 ζ dimer in complex with a 15-amino-acid (in 14-3-3 ζ A, residues 416–430) or a 10-amino-acid (in 14-3-3 ζ B, residues 421–430) peptide of ExoS. The 14-3-3 dimer is shown as a blue ribbon and the ExoS peptides are shown as stick models, with carbon, oxygen and nitrogen atoms coloured in yellow, red and blue, respectively. (B) Stereo view of monomer A of 14-3-3 ζ complexed to 15 amino acids of ExoS (416–430). Colour coding is as in panel A. An omit electron density map of the ExoS peptide contoured at 3 σ is shown in red.

(Figure 1A). In Figure 1B, we show the omit map of the ExoS peptide bound to monomer A of 14-3-3 ζ (14-3-3 ζ A) contoured at 3 σ . The peptide binds in an extended conformation and occupies more than two-thirds of the 14-3-3 binding groove.

The interaction of ExoS and 14-3-3 differed from that observed for other known partners with 14-3-3 in several important ways. In contrast to the structures already elucidated for the 14-3-3 consensus motifs I–III, the binding motif of ExoS is not coordinated predominantly by electrostatic interactions. Rather, ExoS is bound to 14-3-3 mainly via hydrophobic contacts between leucines 422, 423, 426 and 428, which form an extensive hydrophobic interaction surface with the ‘roof’ of the amphipathic groove constituted by the 14-3-3 residues Pro165, Ile166, Leu216, Ile217, Leu220 and Leu172 (Figure 2). In addition, the peptide is coordinated by several H-bonds involving His418, Asp424 and Asp427 of ExoS peptide and Asp213, Lys49 and Tyr 128 of 14-3-3 ζ (Figure 2). The most important feature of the high-affinity binding of the consensus motifs I–III to 14-3-3 is the accommodation of a phosphorylated residue by a basic cluster of 14-3-3 residues consisting of Lys49, Arg56 and Arg127. Thus, it was of interest to analyse whether this part of 14-3-3 also contributes significantly to binding of ExoS. Our crystal analysis shows that the side chain of Asp427 is too far away from the basic cluster (4.8 Å from Lys49, 5.73 Å from Arg127 and 5.48 Å from Arg56) for significant interaction and that Asp424 only contacts Lys49 (Figure 3). This suggests that the important attractive forces resulting in ExoS binding to 14-3-3 are contributed by the hydrophobic contacts of

leucines 422, 423, 426 and 428 of ExoS and that electrostatic interactions between ExoS and 14-3-3 via aspartates 424 and 427 are of secondary importance.

The binding mode of ExoS to 14-3-3 differs with respect to the nature of the main binding forces and the general geometry of binding. Previously observed 14-3-3/client complexes display a binding mode that—regarding their orientation from N- to C-terminus—is opposite to the ExoS binding mode. The corresponding 14-3-3 binding motif from serotonin-*N*-acetyltransferase AANAT (Obsil *et al*, 2001) crosses the amphipathic groove as shown in Figure 4A in an N- to C-terminal direction from right to left. However, ExoS is orientated in an antiparallel fashion to this, starting with the N-terminal part from left to right (Figure 4A). Whereas the ‘entry point’ of the AANAT N-terminus and the ExoS C-terminus is located at nearly exactly the same position, the other ends of the polypeptide chain emerge at different locations; ExoS leaves the groove at one far end while keeping close contact to 14-3-3, whereas the AANAT peptide leaves the groove perpendicular to the groove’s base after the proline-induced kink in its polypeptide chain (Figure 4A and B). These features indicate that ExoS binds 14-3-3 in a manner distinct from modes I–III; we therefore designate this novel phosphorylation-independent binding geometry as mode IV.

Leucines 422, 423 and 428 are important determinants for the induction of cell death by the ADP-ribosylating domain of ExoS in cell culture

Based on this novel insight provided by our new 14-3-3/ExoS complex crystal structure, we wished to confirm the functional relevance of these molecular contacts by examining ExoS-mediated cytotoxicity. For these experiments, we utilized *Yersinia pseudotuberculosis* bacteria as vehicles for the delivery of ExoS into mammalian cells. The type III secretion system of *Y. pseudotuberculosis* is similar to that of *P. aeruginosa* and has previously been shown to efficiently translocate *P. aeruginosa* type III effector proteins into host cells (Frithz-Lindsten *et al*, 1997). HeLa cells were infected for 2 h with *Y. pseudotuberculosis* bacteria engineered to secrete and translocate either the wild type or mutant variants of ExoS into the target cells, in an arabinose-inducible manner (Henriksson *et al*, 2002b). Assessment of bacterial growth of each strain by viability counts, both during initial infection and also after extended infection, indicated that the same bacterial load was present in each assay (data not shown). Following induction with arabinose, HeLa cells incubated with bacteria secreting each of the examined ExoS variants initially showed cell rounding and substantial amounts of cell detachment. These morphological changes have been shown to result from ExoS’s GAP activity (Frithz-Lindsten *et al*, 1997; Kaufman *et al*, 2000; Henriksson *et al*, 2000a) and indicate that the mutant toxins folded correctly and were successfully injected into the HeLa cells. Cell death was assessed by Trypan blue exclusion assays 24 h after infection (Figure 5A). In agreement with previously published results, infection with wild-type ExoS, ExoS(D424A:D427A) and ExoS(L426A) mediated a non-reversible change in cell morphology and ultimately cell death (Yasmin *et al*, 2006). Approximately, only 11–14% of infected cells survived (Figure 5A, lanes 3, 7 and 9). In contrast, over 90% of the cells survived upon infection with bacteria expressing ExoS(LDL426-428AAA) and ExoS(L428A) mutants (Figure 5A, lanes 4 and 8) (Yasmin *et al*, 2006).

Figure 2 Residues 416–430 of ExoS binding to 14-3-3 ζ . (A) Binding of ExoS (416–430) to 14-3-3 ζ . The 14-3-3 protein is shown as blue surface with residues contacting ExoS leucines 422, 423, 426 and 428 coloured in green. ExoS (416–430) is shown as sticks with carbon, nitrogen and oxygen atoms coloured in yellow, blue and red, respectively. Important residues of 14-3-3 ζ contacting ExoS are labelled white and the corresponding residues in ExoS are labelled yellow. Leucines 422, 423, 426 and 428 of ExoS constitute an extensive hydrophobic interaction surface with the nonpolar ‘roof’ of the 14-3-3 binding groove consisting of residues Pro165, Ile166, Leu172, Leu216, Ile217 and Leu220. (B) Schematic representation of the interface between 14-3-3 ζ and ExoS (418–429). Residues from ExoS are shown as sticks coloured as in panel A. Residues from 14-3-3 are represented as blue-filled squares. Arrows indicate polar interactions.

The structural crystal analysis also identified residues L422 and L423 as important determinants for the interaction between 14-3-3 and ExoS. Consistent with this finding, cells infected with bacteria that produced and translocated ExoS(L422A) and ExoS(L423A) showed a partial defect in cell killing, with survival of 59 and 54% of cells, respectively (Figure 5A, lanes 5 and 6). Thus, the measurement of cytotoxicity in ExoS-intoxicated cells supports our structural observations that leucines at positions 422, 423 and 428 are important determinants for the binding of 14-3-3 to ExoS and subsequent activation of the ADP-ribosyltransferase activity of this toxin. To exclude the possibility that differences in cytotoxicity were caused by variable expression or secretion of the ExoS variants, we examined the amounts of ExoS in

whole-cell lysates and in culture supernatants of *Y. pseudotuberculosis* strains grown in calcium-depleted medium containing arabinose. We confirmed equivalent levels of ExoS expression and secretion by each strain (Figure 5C and D). Thus, Leu422, Leu423 and Leu428 are required for ExoS’s cytotoxicity.

ExoS has been postulated to exert its virulence effects by intoxicating macrophages and preventing these cells from engulfing and eradicating *P. aeruginosa* (Rocha *et al*, 2003). For this reason, we also tested the cytotoxic properties of the ExoS variants in J774A.1 cells, a murine macrophage-like cell line. Comparable results as above were obtained upon infection of these cells with ExoS-secreting *Y. pseudotuberculosis* bacteria. Infection with wild-type ExoS, ExoS(L426A) and

Figure 3 Role of the phosphopeptide coordinating basic pocket of 14-3-3 in ExoS binding. (A) $2F_o - F_c$ electron density map contoured at 2σ showing the basic pocket of 14-3-3 ζ and neighbouring 14-3-3 residues as well as the ExoS peptides and several coordinated water molecules. (B) Superimposition of the mode I consensus 14-3-3 binding peptide (magenta carbons) (PDB code 1QJB) and ExoS (yellow carbons) and selected residues from 14-3-3 ζ , in complex with either mode I (green carbons) or ExoS (blue carbons). Electrostatic interactions of less than 3.5 Å are shown as dotted lines. The positions of Asp424 and Asp427 are too remote from the basic pocket of 14-3-3 ζ (Arg56, Arg127, Lys49 and Tyr128) to mimic the binding of the phosphorylated amino acid of the mode I consensus peptide. In addition, the triangular cluster of Lys49, Arg56 and Arg127 is disrupted by a significant side-chain movement of Lys49 in the ExoS complex structure.

Figure 4 Comparison of binding of ExoS and serotonin-N-acetyl transferase (AANAT) to 14-3-3. (A) The ExoS peptide is coordinated by 14-3-3 ζ in an opposite orientation relative to AANAT. AANAT and also all other structurally known 14-3-3 interactors are coordinated in an N- to C-terminal direction starting from the phosphoresidue binding basic pocket to the exit point of the groove. In contrast, ExoS is coordinated from the C- to N-terminus starting from the equivalent position in the basic pocket. Both peptides are coloured in rainbow (blue, N-terminus; red, C-terminus). For clarity, only the main-chain atoms are shown. (B) Comparison of the exit/entry points of ExoS and the AANAT peptide. Both peptides are bound in elongated conformation. With AANAT, the proline at position +2 after the phosphorylated threonine serves the exit of the peptide from the 14-3-3 groove. In contrast, the ExoS peptide maintains intimate contact to the 14-3-3 binding groove before exiting the 14-3-3 dimer.

ExoS(D424A:D427A) resulted in significant amounts of cell death (Figure 5B, lanes 3, 7 and 9), whereas cells survived infection with ExoS(LDL426-428AAA) and ExoS(L428A) (Figure 5B, lanes 4 and 8). As was observed in HeLa cells, intoxication with ExoS(L422A) and ExoS(L423A) caused a partial reduction in cell survival (Figure 5B, lanes 5 and 6).

ADP-ribosylation of the small GTP-binding proteins RAS and RAP1 by ExoS variants

To further examine the biological importance of leucine residues 422, 423 and 428 for the ADP-ribosylating activity of ExoS, we investigated the ability of these mutants to modify known endogenous targets, such as the small GTP-

Figure 5 Cytotoxicity and host cell modification following infection of mammalian cells with ExoS variants. (A) Viability of HeLa cells 24 h after intoxication with ExoS variants. HeLa cells were infected for 2 h in the presence of 0.1 % arabinose with *Yersinia* (YPIII) carrying plasmids expressing different variants of ExoS. Lanes 1 and 2, uninfected cells; lane 3, YPIII(pMF384) expressing wild-type ExoS(wt); lane 4, YPIII(pMF516) expressing ExoS(LDL426-428AAA); lane 5, YPIII(pMF697) expressing ExoS(L422A); lane 6, YPIII(pMF662) expressing ExoS(L423A); lane 7, YPIII(pMF582) expressing ExoS(L426A); lane 8, YPIII(pMF583) expressing ExoS(L428A), lane 9, YPIII(pMF523) expressing ExoS(D424A:D427A). Both loose and semi-detached cytotoxic cells were washed free from bacteria and transferred to a new Petri dish and incubated overnight with medium containing gentamicin. A Trypan blue exclusion assay was performed 24 h after infection to quantitate the percentage of dead cells. Error bars represent s.e.m. $\pm$. (B) Viability of J774 cells 24 h after intoxication with ExoS variants. J774 cells were infected with *Yersinia* (YPIII) expressing variants of ExoS as described in panel A. (C) ExoS secretion and (D) expression after each *Yersinia* strain was induced in the presence of arabinose and analysed using anti-ExoS antibodies. (E–I) ExoS-mediated ADP-ribosylation of HeLa cell proteins following infection and, before harvest, stimulation with EGF (50 ng/ml) for 5 min. HeLa cells were lysed and Western blot analysis was performed on immunoblotted filters with anti-RAS (D), anti-RAP1 (F), anti-phospho-ERK1/2 (G), anti-phospho-PKB/Akt (H) and anti-pan-PKB/Akt (I) antibodies.

binding proteins RAS and RAP1 and their ability to impair downstream signalling cascades. ExoS variants were produced and delivered into eukaryotic cells by genetically modified *Y. pseudotuberculosis* (Henriksson *et al*, 2002b). After 60 min of infection, HeLa cells were stimulated with EGF for 5 min, harvested and then lysed. The resultant lysate

was separated by SDS-PAGE followed by immunoblotting with anti-RAS, anti-RAP1, anti-phospho-ERK and anti-phospho-PKB/Akt (Figure 5E–H). An increase in the apparent size of RAS and RAP1, indicative of the addition of an ADP-ribose group, was observed in cells infected with bacteria expressing all ExoS variants, except when infected with ExoS(L428A) or

ExoS(LDL426-428AAA) (Figure 5E and F, lanes 4 and 8). These latter two variants did not exhibit any detectable ADP-ribosylation ability. This correlates well with our cell survival observations. Interestingly, both ExoS(L422A) and ExoS(L423A), which were partially impaired in their ability to kill mammalian cells, were also unable to modify RAS and RAP1 as efficiently as wild-type ExoS (Figure 5E and F, compare lane 3 with lanes 5 and 6). These observations correlated well with the results of cell survival assays.

Activation of RAS normally results in phosphorylation of the downstream signalling molecules ERK and PKB/Akt (Henriksson *et al*, 2000a). We therefore examined whether ADP-ribosylation of RAS by ExoS resulted in functional inactivation of RAS and loss of ERK and PKB/Akt phosphorylation. Whereas infection of cells with bacteria expressing wild-type ExoS markedly prevented phosphorylation of ERK and PKB/Akt (Figure 5G and H, lane 3), ExoS(LDL426-428AAA) and ExoS(L428A) caused near-normal levels of phosphorylation (Figure 5G and H, lanes 4 and 8). ExoS(L426A) and ExoS(D424A:D427A) inhibited phosphorylation similarly to wild-type ExoS, indicating that these residues were not critical for ExoS activity. ExoS(L422A) and ExoS(423A) partially inhibited phosphorylation of PKB/Akt (Figure 5G and H, lanes 5 and 6). Thus, variants of ExoS unable to modify RAS and RAP1 were also unable to inhibit phosphorylation of PKB (compare Figure 5E and F with G and H). These observations lend further credence to our structural analysis, whereby those residues predicted to make contacts with the 14-3-3 cofactor are also important for the functional capability of the ExoS toxin.

Residues 426, 427 and 428 of ExoS are necessary for virulence in a mouse model of pneumonia

To determine the role of the 14-3-3/ExoS interaction in a more clinically relevant model, we employed a clinical isolate of *P. aeruginosa* (Shaver and Hauser, 2004). A mutant of PA99 (designated PA99null) that expresses a functional type III secretion system but does not secrete any known effector proteins was used as a means to deliver variants of ExoS into mice. Alleles of the *exoS* gene were inserted into a neutral site of the PA99null chromosome to yield isogenic strains that secreted the following ExoS variants: wild-type ExoS, ExoS(E379A/E381A), an ExoS variant harbouring E379A/E381A, substitutions that directly impair ADP-ribosyltransferase activity (Pederson and Barbieri, 1998), and ExoS(LDL426-428AAA), which is defective in its ability to interact with 14-3-3 proteins. These strains were designated PA99null + S, PA99null + S(E379A/E381A) and PA99null + S(LDL426-428AAA), respectively. All mutants grew at rates similar to those of wild-type strain PA99 (data not shown) and each secreted appropriate amounts of the ExoS variant when grown under secretion-inducing conditions (data not shown).

These mutant strains were used to examine the biological importance of ExoS residues L426, D427 and L428 to *P. aeruginosa* pathogenesis in a mouse model of acute pneumonia caused by *P. aeruginosa* (Shaver and Hauser, 2004). Mice were inoculated intranasally with 1.2×10^6 CFU of bacteria, and bacterial loads in the lung of individual animals were measured after 18 h. At this time point, 2.9×10^5 CFU of bacteria were observed in the lungs of mice infected with *P. aeruginosa* expressing wild-type ExoS (Figure 6A). Consistent with previous reports (Shaver and Hauser,

2004), PA99null, which does not secrete ExoS, and PA99null + S(E379A/E381A), the ADP-ribosyltransferase mutant, were rapidly cleared, with only 1.2×10^4 and 1.8×10^4 CFU, respectively, remaining in the lungs of these mice after 18 h. Interestingly, PA99null + S(LDL426-428AAA) was cleared similarly to PA99null + S(E379A/E381A), with only 1.9×10^4 CFU remaining in the lungs after 18 h.

To further assess the virulence associated with residues of ExoS important in binding of 14-3-3, we measured the survival of infected mice. Mice were inoculated with 9.2×10^6 CFU (approximately two times the 50% lethal dose for mice infected with bacteria secreting wild-type ExoS) of each ExoS mutant and monitored for survival over 7 days. As previously reported, infection with PA99null + S, the strain secreting wild-type ExoS, resulted in 100% mortality after approximately 36 h, and infection with PA99null resulted in survival of all mice (Shaver and Hauser, 2004). PA99null + S(LDL426-428AAA) affected survival similarly to PA99null + S(E379A/E381A), the ADP-ribosyltransferase mutant. In both cases, mice began to die at 72 h post-infection, and approximately 50% of mice had died after 7 days of infection (Figure 6B). This implies that 426-LDL-428 is required for an interaction with 14-3-3 proteins and subsequent virulence in acute pneumonia.

Together, these data indicate that substitution of L426, D427 and L428 is critical for the virulence of ExoS. Substitutions of these residues resulted in decreased virulence similar to that observed following abrogation of ExoS's ADP-ribosyltransferase activity. Thus, substitution of residues predicted to be crucial by structural analysis for binding of ExoS to 14-3-3 resulted in the loss of ability of 14-3-3 to activate the ADP-ribosyltransferase activity of ExoS *in vivo*. This in turn caused a decrease in virulence associated with ExoS, manifested by clearance of *P. aeruginosa* from the lungs and increased survival of infected mice.

Discussion

It has been generally accepted that phosphorylation-independent interactions between 14-3-3 and its binding partners occur in a manner similar to that of the mode-I binding partner pS-RAF-259. This assumption is based on the finding that a non-phosphorylated artificial ligand for 14-3-3, R18, was co-crystallized with 14-3-3 in a position similar to that of phosphorylated pS-RAF-1-259 (Petosa *et al*, 1998). In the complex structure of 14-3-3- ζ with R18, five residues, comprising the sequence WLDLE were resolved. Here the aspartate (D) and glutamate (E) side chains generated a negative charge density comparable to that of the phosphoserine of qpS-RAF-1-259 (Petosa *et al*, 1998). R18 has the ability to impair the ADP-ribosylation activity of ExoS of *P. aeruginosa* (Masters *et al*, 1999; Henriksson *et al*, 2000a); so it was proposed that pS-RAF-1-259 and ExoS most likely share a common binding site on 14-3-3. However, the prediction that phosphorylation-independent interactions are mediated by acidic residues had to be questioned following the observation that substitution of both negatively charged aspartate residues at positions 424 and 427 of the defined binding motif of ExoS towards 14-3-3 was not essential for the interaction with 14-3-3 or for enzymatic activation of ExoS (Yasmin *et al*, 2006). Therefore, it was necessary to solve the crystal structure of the contact surface between 14-3-3 and ExoS.

Figure 6 Virulence of ExoS containing substitutions predicted to affect binding to 14-3-3, as measured in a mouse model of acute pneumonia. (A) Bacterial load in the lungs of mice infected with *P. aeruginosa* secreting ExoS variants. Each symbol represents the bacterial load in the lung of an individual animal at 18 h post-infection. Bars represent the median values. Bacterial loads associated with infection with PA99null + S(LDL426-428AAA) differed significantly from those associated with PA99null + S ($P < 0.05$, Student's *t*-test). (B) Survival of mice infected with *P. aeruginosa* secreting ExoS variants. Data points represent the number of animals surviving in each experimental group over time ($n = 10$ mice per strain, pooled from two separate experiments). Survival of mice infected with PA99null + S(LDL426-428AAA) differed significantly from that of mice infected with PA99null + S ($P < 0.0001$ log-rank test for equality of survivor functions).

From our crystal structure analysis of 14-3-3 and ExoS, we suggest that the main attractive forces between ExoS and 14-3-3 are of hydrophobic nature and suggest that the electrostatic binding mediated by Asp424 and Asp427 only marginally contributes to overall binding. Four leucine residues in the 14-3-3 binding motif of ExoS (Leu422, Leu423, Leu426 and Leu428) constitute a hydrophobic patch that makes contact with the hydrophobic 'roof' of the amphipathic groove of 14-3-3, consisting of residues Pro165, Ile166, Leu216, Ile217, Leu220 and Leu172 (Figure 2). As confirmed by our mutational analysis, the electrostatic interactions between Asp424 and Asp427 of ExoS and Lys49 and Tyr128 of 14-3-3, respectively, do not appear strong enough to contribute significantly to the overall binding energy. In

particular, the suggested mimicry of the phosphorylated residue in the mode I-III binding (phosphoserine or phosphothreonine) motifs by Asp424 and Asp427 can be excluded due to our structure.

The binding of phosphopeptides to 14-3-3 is dominated by polar contacts between main-chain atoms of the peptide ligand with 14-3-3 residues. For example, in the co-crystal structure of Rittingers' mode-1 peptide (Rittinger *et al*, 1999; PDB code 1QJB) with 14-3-3 ζ we find three polar contacts of 14-3-3 to the backbone of the peptide: Lys49 to the carbonyl oxygen of the +2 residue, Asn173 to the backbone amide nitrogen of the +1 residue and Asn224 to the backbone amide nitrogen and the main-chain carbonyl oxygen of the -1 residue. In addition to the coordination of the phosphate

group of the peptide by the basic cluster of 14-3-3 (Lys49, Arg56 and Arg127) there is only one side-chain contact between 14-3-3 (Asn224 and Trp228) to the phosphopeptide. In contrast to this, in our structure we find two main-chain contacts of the ExoS (Gly419 and Ala429) to 14-3-3 (Asp213 and Asn173) and eight side-chain contacts (Figure 2B). Interestingly, the contact between the peptides main chain to 14-3-3s Asn173 can be found in both modes.

Our analysis suggests that there may exist three basic mechanisms by which 14-3-3 proteins interact with their binding partner. As mentioned above, the coordination of the consensus mode I–III motifs significantly relies on electrostatic binding of the phosphoserine/phosphothreonine residues by a basic cluster of amino acids within the amphipathic groove of 14-3-3. In this cluster, two arginines (Arg56 and Arg127), one lysine (Lys49) and one tyrosine residue (Tyr128) coordinate the phosphate moiety. In this mechanism, phosphorylation of the consensus motif is a prerequisite for high-affinity binding to 14-3-3, whereas unphosphorylated analogues barely bind. A second mechanism has been described for the artificial peptide R18. R18 binds 14-3-3 in a phosphorylation-independent manner via negatively charged residues coordinated by the aforementioned basic cluster. An albeit poorly resolved co-crystal structure of the R18 peptide with 14-3-3 ζ supports this model and demonstrates that secondary carboxyl groups of the aspartate and glutamate residues of R18 substitute for the phosphorylated residues of consensus motifs (Petosa *et al*, 1998). The third mechanism, described in this study and here designated ‘mode IV’, involves the interaction of hydrophobic residues with the roof of the 14-3-3 amphipathic groove. In ExoS, the two aspartate residues Asp424 and Asp427 of ExoS do not mimic a phosphoryl group, as they are too far away from the positions occupied by phosphorylated serine or phosphorylated threonine residues during binding of consensus mode I–III motifs. Additionally, the triangular architecture of the cluster consisting of Lys49, Arg56 and Arg127 of 14-3-3 is disrupted by a significant side-chain movement of Lys49. Although this side-chain movement enables Asp424 to contact Lys49, and although Asp427 establishes an electrostatic contact to Tyr128, this situation is not equivalent to the phosphorylation-mediated binding of the consensus motifs.

Another surprising feature of our structure is the orientation of the ExoS peptide, which is opposite to that of the structurally elucidated binding modes I–III (Figure 4A). The 14-3-3 binding motif of ExoS runs nearly perfectly in an antiparallel fashion relative to that of serotonin-*N*-acetyltransferase. The binding of distinct ligands in opposite orientations is unusual but not unique to 14-3-3 proteins. For example, the polyproline type II helix (PPII helix) of protein partners that bind to SH3 and WW domains can be bound in two possible orientations, suggesting a functional significance (Zarrinpar *et al*, 2003). Likewise, the MIU (motif interacting with ubiquitin) of Rabex-5 (Lee *et al*, 2006; Penengo *et al*, 2006) binds to ubiquitin similar to but in the opposite orientation of other ubiquitin binding motifs such as the UIM (ubiquitin-interacting motif) of Vps 27 (Swanson *et al*, 2003). In contrast to 14-3-3 binding partners, the ubiquitin ligands require the same critical amino acids for interaction, independent of orientation. For example, both the MIU of Rabex-5 and the UIM of Vps 27 display a central alanine (Ala58 and Ala266, respectively) that is bound within

a hydrophobic depression comprised of Leu8, Ile44, His68 and Val70 of ubiquitin. Another protein that binds two ligands in the reverse orientation is Fus3. This MAP kinase binds motifs within the scaffold protein Ste5 and the substrate Far1 in an exact antiparallel manner (Bhattacharyya *et al*, 2006). Here also, important residues of the ligand motifs are conserved and occupy the same sites within the Fus3 binding groove, although the motif backbones are aligned in opposite directions. It will be interesting to see whether other non-phosphorylated 14-3-3 binding motifs also align antiparallel to the phosphorylated consensus motifs I–III, indicating that ExoS may serve as a paradigm for an as yet unrecognized phosphorylation-independent 14-3-3 binding mechanism.

Our crystal structure analysis is supported by biological and functional assays utilizing several approaches. A cell infection model was used to examine the cellular and molecular consequences of specific amino-acid substitutions in the 14-3-3 binding site of ExoS. Replacement of Leu426–Asp427–Leu428 with three alanine residues resulted in a dramatic decrease in the ability of ExoS to ADP-ribosylate two of its substrates, RAS and RAP1, indicating that these amino acids were crucial for binding and activation of ExoS by 14-3-3. Additional studies demonstrated that substitution of Leu428 was responsible for nearly all of this decrease in interaction. Likewise, substitution of Leu422 and Leu423 significantly reduced the modification of RAS and RAP1, indicating that these residues were also important in binding of ExoS by 14-3-3. In contrast, substitution of Asp424 and Asp427 confirmed that these acidic residues played little or no role in this interaction. Examination of RAS activity by measuring phosphorylation of ERK and PKB/Akt, downstream targets of RAS, confirmed the importance of these residues in the ADP-ribosylation of RAS by ExoS. This is further supported by the decreased ability of ExoS(L422A), ExoS(L423A) and ExoS(L428A) to inhibit ERK and PKB/Akt activation upon stimulation with EGF. Examination of cytotoxicity, a cellular consequence of ExoS-mediated ADP-ribosylation, confirmed the importance of these three leucine residues in the binding and activation of ExoS by 14-3-3.

Finally, a panel of isogenic bacterial mutants in a clinical isolate of *P. aeruginosa* (PA99) that naturally secretes type III toxins was used to evaluate carefully the relative role in disease pathogenesis of ExoS residues within the 14-3-3 binding domain. In a mouse model of pneumonia, residues 426–428 were absolutely required for ExoS virulence. This is the first study to conclusively show in an animal model that the virulence associated with ExoS is dependent on its binding and interaction with 14-3-3. In this model system, secretion of ExoS(LDL426-428AAA), which contains substitutions in the 14-3-3 binding site, was associated with increased survival and decreased bacterial burden in the lungs. In fact, the virulence properties of ExoS(LDL426-428AAA) were similar to those of ExoS(E379A-E381A), a catalytically inactive variant of ExoS, again suggesting that substitution of crucial leucines in the 14-3-3 binding site prevented activation of ExoS’s ADP-ribosyltransferase activity.

In conclusion, we suggest that the 14-3-3-ExoS phosphorylation-independent interaction occurs in a novel binding mode (IV) mostly relying on hydrophobic contacts and to a lesser degree electrostatic interactions. A predominant patch of ExoS (residues Leu422, Leu423, Leu426 and Leu428) interacts with the amphipathic ‘roof’ of the groove of 14-3-3

in a geometrically antiparallel manner. This patch of residues is essential for the enzymatic activity by ExoS in both a cell culture and mouse pneumonia model system.

Materials and methods

Crystallization and structure elucidation

Human 14-3-3 ζ was co-produced with GST-ExoS(353–453) in *E. coli* (BL21 DE3) and purified via GSH-Sepharose. After cleavage of the GST-fusion tag with thrombin, the 14-3-3 ζ /ExoS complex was further purified via gel filtration. The purified complex was trypsinized for 15 min at room temperature at a molar stoichiometry of 5000:1. Before aliquotation and freezing, Pefabloc was added to a final concentration of 2 mM. Crystals appeared after 2–4 weeks in 0.1 M Hepes/NaOH, pH 7.8, 20% PEG 3350 and 0.2 M LiSO and grew within 2–4 days to dimensions of $400 \times 200 \times 200 \mu\text{m}$. For flash-freezing, the crystals were incubated in mother liquor supplemented with 30% PEG 3350 and 7% glycerol for 1 min. Data collection was performed at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland Beamline X10SA (PXII) and was processed with XDS (Kabsch, 1993). Molecular replacement was carried out with MOLREP. The high-resolution structure of 14-3-3 ζ (PDB code 1QJA) was used as the search model. The obtained model was subjected to iterative rounds of model building and refinement using the programs COOT and REFMAC (Murshudov *et al*, 1997; Emsley and Cowtan, 2004). Figures were prepared with PYMOL (www.pymol.org).

Coordinates

The atomic coordinates and structure factors of the complex have been deposited in the Protein Data Bank under the ID code 2002.

Cell cultures and cell lysis

HeLa cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine calf serum and 100 U/ml penicillin. Mouse macrophage-like J774A.1 cells were maintained in α -MEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO₂. Following bacterial infection, cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed on ice in lysis buffer (1% (v/v) Triton X-100, 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA supplemented with protease inhibitors (Complete, Roche, Germany)).

Western analysis and antibodies

The antibodies phospho-p42/44 MAPK, phospho-PKB (Ser473), and pan-Akt were obtained from Cell Signaling Technology (Beverly, MA). Anti-14-3-3 β (SC-629) was purchased from Santa Cruz (New York, NY, USA); monoclonal antibodies against RAS (R02120) and RAP1 were obtained from Transduction Laboratories (Lexington, KY, USA). Anti-ExoS was from Agrisera AB, Sweden. Immunoblotting was performed according to the manufacturer's instructions (Pierce, Rockford, IL, USA and ECL Plus, Amersham Biosciences).

Construction of arabinose-inducible ExoS derivatives and infection of cells

To ensure protein stability of full-length ExoS derivatives, mutant alleles were coexpressed in *Y. pseudotuberculosis* with *orf1*, encoding the cognate non-secreted chaperone of ExoS (Yahr *et al*, 1996). In all cases, DNA was amplified by PCR using conditions described previously (Francis and Wolf-Watz, 1998). Construction of pMF384 (ExoS), pMF582 (ExoS_{L426A}), pMF583 (ExoS_{L428A}), pMF523 (ExoS_{PD424:427AA}) and pMF516 (ExoS_{L426-428AAA}) has been described previously (Yasmin *et al*, 2006). pMF697 (ExoS_{L422A}) and pMF662 (ExoS_{L423A}) were obtained by replacing the C-terminal *Clal/KpnI* *exoS* fragment from pMF384 with DNA amplified and digested with *Clal/KpnI* from pGEX-ExoS(L422A) and pGEX-ExoS(L423A), respectively (Yasmin *et al*, 2006), using the *exoS*-specific primers, pexoSseq3 (position 973–991; forward): 5'-AAGTGTATGGCGCTTGGTCT-3' and pexoSd (reverse): 5'-ATGCATGGTACCTCAGGCCAGATCAAGGCCGCG-3'. All constructs were maintained in *E. coli* DH5 and confirmed by sequence analysis using MWG Biotech custom sequencing. Bacterial infection of cells (2×10^6 HeLa cells were infected with 2.6×10^6 bacteria, 2×10^6

J774A.1 cells were infected with 0.6×10^6 bacteria) was performed in the presence of 0.1% L(+)-arabinose as described previously (Henriksson *et al*, 2002b). Loose and semi-detached cytotoxic cells were washed free from the culture dishes and transferred to new Petri dishes and incubated overnight with medium containing gentamicin. A Trypan blue exclusion assay was performed 24 h after infection to quantify the percentage of dead cells.

Construction and growth of *Pseudomonas* mutants

PA99 is a *P. aeruginosa* clinical isolate that naturally encodes the *exoU*, *exoS* and *exoT* genes but lacks the gene for *exoY* (Feltman *et al*, 2001). *E. coli* strains XL1-Blue, GM2163 and TOP10 were used for cloning and propagation of plasmids. *E. coli* strain S17.1 was used for mating of constructs into *P. aeruginosa*. Bacterial strains were streaked from frozen cultures onto Luria-Bertani agar or Vogel Bonner minimal (VBM) agar (Vogel and Bonner, 1956). Antibiotics were used at the following concentrations: ampicillin 50 $\mu\text{g}/\text{ml}$ for *E. coli*; carbenicillin 500 $\mu\text{g}/\text{ml}$, gentamicin 100 $\mu\text{g}/\text{ml}$ and tetracycline 100 $\mu\text{g}/\text{ml}$ for *P. aeruginosa*. PA99null is *P. aeruginosa* strain PA99 in which each of the endogenous effector-encoding genes (*exoS*, *exoT* and *exoU*) has been disrupted, as described previously (Shaver and Hauser, 2004). PA99null + S contains a wild-type allele of the *exoS* gene under control of its endogenous promoter that is inserted at a neutral site in the PA99null chromosome (Shaver and Hauser, 2004). PA99null, expressing ExoS(LDL426-428AAA), was constructed as follows: a 2.0 kb *exoS*-containing *EcoRI*-*EcoRI* fragment from pGS001 was cloned into mini-CTX1 that was previously digested with *EcoRI* to create mini-CTX1-ExoS, as described previously (Shaver and Hauser, 2004). Mutations yielding ExoS(LDL426-428AAA) were introduced into mini-CTX1-ExoS using the Quik-change II XL Site-Directed Mutagenesis approach (Stratagene, La Jolla, CA). The resulting plasmid was conjugated to the *P. aeruginosa* strain PA99null and integrated into the chromosomal *attB* site, according to the method of Hoang *et al* (2000). Thus, ExoS(LDL426-428AAA) was expressed under control of the endogenous *exoS* promoter from a single-copy chromosomal insertion. This strain was designated PA99null + S(LDL426-428AAA). Nucleotide sequencing was performed to verify that the appropriate mutations were generated, and immunoblot analyses of culture supernatants were performed to ensure that ExoS protein was secreted (data not shown).

Mouse model of acute pneumonia

Studies of acute pneumonia were conducted using the mouse model described by Comolli *et al* (1999). Briefly, bacterial cultures were grown overnight in MINS medium at 37°C with shaking (250 r.p.m.) and were then diluted and regrown to exponential phase. Bacteria were collected by centrifugation and resuspended in PBS (Invitrogen) to the appropriate concentration. Six- to seven-week-old female BALB/c mice were anaesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). For survival assays, mice were intranasally inoculated with 9.2×10^6 CFU of bacteria in 50 μl PBS, as determined by optical density. Inocula were confirmed by plating of serial dilutions onto VBM agar. Survival was monitored for 7 days after infection. Mice were killed when severe illness developed and were scored as dead. The experiment was performed twice and results were pooled. For determination of bacterial numbers in lungs, mice were intranasally inoculated with 1.2×10^6 CFU of bacteria in 50 μl PBS and re-anaesthetized and killed at 18 h post-infection. Lungs were aseptically removed and homogenized in 5 ml PBS. Bacterial load in each organ was determined following plating of serial dilutions on VBM agar and incubation at 37°C for 24 h. All experiments were approved by and performed in accordance with the guidelines of the Northwestern University Animal Care and Use Committee.

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