Interaction of 14-3-3 with a Nonphosphorylated Protein Ligand, Exoenzyme S of *Pseudomonas aeruginosa*†

Shane C. Masters,‡ Kristin J. Pederson,§ Lixin Zhang,|| Joseph T. Barbieri,§ and Haian Fu*,||

Graduate Program in Molecular Therapeutics and Toxicology and the Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322, and the Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226-0509

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ABSTRACT: The 14-3-3 proteins are a family of conserved, dimeric proteins that interact with a diverse set of ligands, including molecules involved in cell cycle regulation and apoptosis. It is well-established that 14-3-3 binds to many ligands through phosphoserine motifs. Here we characterize the interaction of 14-3-3 with a nonphosphorylated protein ligand, the ADP-ribosyltransferase Exoenzyme S (ExoS) from *Pseudomonas aeruginosa*. By using affinity chromatography and surface plasmon resonance, we show that the zeta isoform of 14-3-3 (14-3-3ζ) can directly bind a catalytically active fragment of ExoS in vitro. The interaction between ExoS and 14-3-3ζ is of high affinity, with an equilibrium dissociation constant of 7 nM. ExoS lacks any known 14-3-3 binding motif, but to address the possibility that 14-3-3 binds a noncanonical phosphoserine site, we assayed ExoS for protein-bound phosphate by using mass spectrometry. No detectable phosphoproteins were found. A phosphopeptide ligand of 14-3-3, pS-Raf-259, was capable of inhibiting the binding of 14-3-3 to ExoS, suggesting that phosphorylated and nonphosphorylated ligands may share a common binding site, the conserved amphipathic groove. It is conceivable that 14-3-3 proteins may bind both phosphoserine and nonphosphoserine ligands in cells, possibly allowing kinase-dependent as well as kinase-independent regulation of 14-3-3 binding.

The 14-3-3 family consists of several dimeric, highly conserved, broadly expressed eukaryotic proteins. 14-3-3 proteins are primarily known for the large, diverse group of signaling molecules that interact with them. A few examples include kinases such as Raf (*1*−*4*), phosphatases such as cdc25 (*5*), and molecules involved in apoptosis, such as Bad (*6*). Despite the number of proteins known to interact with 14-3-3, the physiological function of this family has not been well-established. Genetic studies in yeast, which possess only two isoforms, argue for an important physiological role for 14-3-3. Deletion of both isoforms causes death (*7*−*9*) or slow growth and impairment of multiple signaling pathways (*10*), depending on the strain of yeast used.

14-3-3 is capable of stimulating the activities of several enzymes, including tyrosine and tryptophan hydroxylases (*11*), and exoenzyme S (ExoS[1]) from *Pseudomonas aeruginosa*. ExoS is an ADP-ribosyltransferase that catalyzes the transfer of an ADP-ribose moiety from NAD^+ to substrate proteins, notably small G proteins such as Ras (*12*). ExoS is dependent on the presence of 14-3-3 for its activity (*13*), but it is not known whether 14-3-3 interacts with ExoS, its substrates, both, or neither. Mutagenesis of 14-3-3 suggests that 14-3-3 may bind to ExoS (*14*); nevertheless, direct evidence for this interaction is lacking. The pathophysiological relevance of ExoS is not fully understood, but studies have shown that *Pseudomonas* strains expressing ExoS are significantly more virulent than isogenic strains lacking ExoS (*15*, *16*). Recent work has demonstrated that ExoS is toxic to eukaryotic cells (unpublished results; refs *16*, *17*).

The mechanism by which 14-3-3 proteins bind their ligands has been extensively studied. Crystallization of the ζ (*18*) and τ (*19*) isoforms of 14-3-3 revealed the presence of a conserved groove in each monomer. This groove has amphipathic properties, with a hydrophobic face on one side and a positively charged face on the other. The amphipathic groove was proposed to be the ligand binding site on 14-3-3 (*18*). Structure-based mutational analysis of 14-3-3 (*14*, *20*), as well as cocrystallization of 14-3-3 with peptide ligands (*21*, *22*), has supported this hypothesis.

The model that 14-3-3 binds ligands via its conserved amphipathic groove implies that these diverse ligands contain a common interacting epitope. One feature observed in many

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* To whom correspondence should be addressed: Department of Pharmacology, Emory University School of Medicine, 1510 Clifton Road NE, Atlanta, GA 30322. Tel: 404-727-0368; Fax: 404-727-0365. E-mail: haianfu@bimcore.emory.edu.

‡ Graduate Program in Molecular Therapeutics and Toxicology, Emory University School of Medicine.

§ Medical College of Wisconsin.

|| Department of Pharmacology, Emory University School of Medicine.

[1] Abbreviations: CT/LT, cholera toxin/heat labile enterotoxin; ESI-MS, electrospray ionization mass spectrometry; ExoS, exoenzyme S from *Pseudomonas aeruginosa*; His_6-ΔN222, His_6-tagged 222 amino acid C-terminal catalytic domain of ExoS; NTA, nitrilotriacetic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography; SPR, surface plasmon resonance.

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14-3-3 interacting proteins is the presence of phosphoserine (*23*). The importance of phosphorylation for 14-3-3 binding was initially observed for tryptophan hydroxylase (*24*) and Raf-1 (*25*). A 14-3-3 binding motif of R-S-x-pS-x-P, where pS is phosphoserine and x is any amino acid, was defined on the basis of the 14-3-3 binding epitope in Raf-1 (*23*). Yaffe et al. have since shown that there is a family of similar motifs that can serve to bind 14-3-3 (*21*). These motifs share the absolute requirement of serine phosphorylation for high-affinity binding. Indeed, many 14-3-3 ligands employ this type of interaction, including Raf-1 (*23*) and Bad (*6*, *26*), and there are also variant phosphoserine motifs used by ligands such as keratin 18 (*27*) and Cbl (*28*). Nonphosphorylated epitopes distant from the phosphoserine core likely also contribute to 14-3-3/ligand interaction, and this has been examined in the platelet glycoprotein Ib-IX-V system (*29*). In a very few cases, such as for the Raf-1 cysteine-rich domain, 14-3-3 may bind to ligands via a mechanism not involving phosphoserine, though in this case it is also possible that the cysteine-rich domain forms part of the serine-259 binding epitope in intact Raf-1 (*30*).

Here we show that 14-3-3 and ExoS interact in a direct fashion, and provide evidence that this interaction does not require phosphoserine. Experiments using phosphoserine 14-3-3 ligands as competitors argue that ExoS binds in the amphipathic groove of 14-3-3. ExoS may represent a novel class of 14-3-3 ligands, those that are not phosphoserine-dependent. The binding of 14-3-3 proteins to nonphosphorylated ligands could have important implications for 14-3-3 signaling. Prototypical phosphoserine-type ligand binding is regulated by protein kinase and phosphatase activity. On the other hand, binding of 14-3-3 to nonphosphorylated ligands may be constitutive or regulated in a kinase-independent manner.

MATERIALS AND METHODS

Protein Expression and Purification. 14-3-3 wild-type and mutant proteins were expressed as His_6-tagged fusions in *Escherichia coli* from pHAF612 (*13*), which encodes 14-3-3ζ in pET15b (Novagen), and purified on Ni^{2+} charged iminodiacetic acid Sepharose beads as previously described (*14*). The His_6 tag was removed from the 14-3-3 proteins by digestion with thrombin. Thrombin and the cleaved His_6 tag were removed by passage over a hybrid column containing Ni^{2+} charged and benzamidine resins. A final purification step over a MonoQ anion-exchange column on an FPLC system was performed to yield the final product. ExoS was expressed in *E. coli* from a pET15b construct (pΔN222) coding for the catalytic domain of ExoS (residues 232−453) and purified on Ni^{2+} charged beads in the same manner as the 14-3-3 proteins. Protein concentrations were estimated using the method of Bradford against a bovine serum albumin standard.

Peptides. R18 (PHCVPRDLSWLDLEANMCLP) and pS-Raf-259 (LSQRQRSTpSTPNVHMV) were synthesized and purified as described (*22*). R18 is a peptide isolated from a phage display library selected against 14-3-3τ that binds 14-3-3ζ with 89 nM affinity (*22*). pS-Raf-259 peptide represents one of three putative 14-3-3 binding sites on Raf-1; this peptide binds 14-3-3ζ with an affinity of 122 nM (*23*).

ExoS Activation Assay. Stimulation of ExoS ADP-ribosyltransferase activity by 14-3-3 was measured essentially as described (*14*). Reactions containing 30 μM [^{32}P]NAD^+, 30 μM soybean trypsin inhibitor, 20 nM ExoS, and varying concentrations of 14-3-3 proteins were made in a 20 μL volume. After incubation at room temperature for 20 min, 12 μL from each sample was spotted onto trichloroacetic acid saturated filter paper. The filter paper was washed and dried, and [^{32}P]ADP-ribose incorporation was determined by liquid scintillation counting. This assay was used to ensure that ExoS proteins used in the binding assays were catalytically active.

Phosphatase Treatment of ExoS. ExoS was diluted to 0.2 g/L in 100 μL of 1 mM $MgCl_2$, 50 mM HEPES, pH 7.5, and incubated for 10 min at 30 °C. Thirty units of calf intestinal alkaline phosphatase was added, and incubation at 30 °C was continued for 60 min. A mock sample without phosphatase was processed simultaneously to control for ExoS stability.

ExoS/14-3-3ζ Affinity Chromatography. Equivalent moles (0.4 nmol) of 14-3-3ζ and His_6-ΔN222 were mixed in 10 mM Tris-HCl, 20 mM NaCl, pH 7.6, and added to 50 μL of Ni^{2+} charged resin. After a 20 min incubation at room temperature, samples were centrifuged at 15000*g* for 3 min. The supernatant was removed, and the resin was washed twice with ice-cold wash buffer (5 mM imidazole, 0.5 M NaCl, 40 mM Tris-HCl, pH 7.9). Bound proteins were eluted with three incubations in ice-cold elution buffer (0.5 M imidazole, 0.25 M NaCl, 10 mM Tris-HCl, pH 7.9). Unbound, wash, and elution fractions were subjected to SDS−PAGE. Gels were fixed and stained with Coomassie Blue. Protein concentrations were normalized to a known concentration of bovine serum albumin using an AMBIS optical imaging system. Protein recoveries were between 50% and 90% of the applied material.

Surface Plasmon Resonance Binding Studies. All studies were performed on a Biacore 2000 instrument (Biacore) using a Ni^{2+} charged nitrilotriacetic acid (NTA) sensor chip. A two buffer system was used per manufacturer recommendations: dispenser buffer was HBS (150 mM NaCl, 10 mM HEPES, pH 7.5) with 3.4 mM EDTA, and running buffer was HBS with 50 μM EDTA. For binding experiments, all four flow cells of an NTA sensor chip were charged with 500 μM $NiSO_4$ in running buffer, and then His_6-ΔN222 was immobilized to different levels in 3 flow cells. A fourth flow cell lacking ExoS was used as a blank. Various concentrations (20−500 nM) of untagged 14-3-3ζ were flowed across all 4 cells for 5 min at 25 μL/min, followed by running buffer or R18 in running buffer. The R18 was used in some runs to prevent mass transport artifacts during dissociation. Comparison of runs made with and without R18 showed very little effect on derived kinetic parameters. After each run, the sensor chip was regenerated with EDTA per manufacturer instructions. Blank subtracted data were fit to a one to one binding model ($A + B \rightleftarrows AB$) using BIAevaluation 2.1 software per manufacturer's instructions. Despite the possibility that one 14-3-3 dimer may bind two ligands simultaneously, this model should be valid due to the low density of ExoS on the surface, causing a 1:1 ExoS/14-3-3 dimer to be the predominant species. The parameters obtained from this analysis were k_{on}, the association rate constant, and k_{off}, the dissociation rate constant.

Electrospray Ionization Mass Spectrometry (ESI-MS). His_6-ΔN222 was fractionated by reversed-phase high-pres-

Table 1. Direct Binding of 14-3-3ζ to Exoenzyme S as Measured by Ni^{2+} Affinity Chromatography[a]

protein	% free	% bound
His-ΔN222	14.1 ± 8.0	86.0 ± 8.0
14-3-3ζ	99.4 ± 0.7	0.7 ± 0.7
14-3-3ζ K49E	100.0 ± 0.0	0.0 ± 0.0[b]
egg albumin	100.0 ± 0.0	0.0 ± 0.0[b]

protein added with His-ΔN222	bound protein (mol/mol of His-ΔN222)
14-3-3ζ	0.91 ± 0.05
14-3-3ζ K49E	0.09 ± 0.00
egg albumin	0.07 ± 0.06

[a] Affinity chromatography was performed as described in the Methods section using either individual proteins (top) or His_6-ΔN222 incubated with the indicated protein in a 1:1 molar ratio (bottom). Results shown are the average of two independent determinations ± standard deviation. [b] Below detection limit of AMBIS optical imaging system.

sure liquid chromatography (RP-HPLC) on a Jupiter C4 silica column (Phenomenex), and material absorbing at 205 nm was collected and pooled. The pooled His_6-ΔN222 (200 pmol) or a positive control protein, bovine β-casein (50 pmol), was diluted into 50 μL of 50 mM NH_4HCO_3, pH 8.0, and digested with 0.5 μg of sequencing grade alkylated trypsin (Promega) for 20 h at 37 °C. The resulting mixtures were then subjected to ESI-MS on a model API 3000 triple quadrupole mass spectrometer (PE-Sciex) equipped with a TurboIonSpray pneumatically assisted electrospray source and MassChrom version 1.1 software. The presence of phosphopeptides in the digest was assessed using the precursor ion scanning mode method (*31*). The experimental conditions were as follows: collision voltage was 120 eV, collision gas flow relative rate was 5, Q3 was set at −79 amu while Q1 was scanned from 300 to 1500 amu in 0.2 amu increments, and dwell time and pause were both 2.0 ms.

Peptide Binding Inhibition Studies. 14-3-3ζ (100 nM) was incubated with various concentrations of either R18 or pS-Raf-259 peptides in running buffer for 1 h. These samples were flowed over an ExoS coated surface as described above. The initial binding event was fit to the equation $R = R_{ss}(1 - \exp(-k_s(t - t_0)))$, where R is the response at time t, R_{ss} is the steady-state response, and t_0 is the time of injection start. The term k_s is equal to $k_{on}C + k_{off}$, and experimental values for k_{on} and k_{off} in the absence of peptide were used to derive an apparent concentration of 14-3-3ζ in the system.

RESULTS

14-3-3ζ and ExoS Bind Directly with High Affinity. A Ni^{2+} affinity chromatographic analysis was designed to determine whether direct, specific binding occurred between ExoS and 14-3-3ζ proteins. A C-terminal 222 residue fragment of ExoS, ΔN222, was chosen for this analysis because it possesses the entire 14-3-3-dependent ADP-ribosyltransferase domain of ExoS (*32*). Conditions were established which allowed the efficient binding of His_6-ΔN222 to Ni^{2+} affinity resin without appreciable nonspecific binding of the non-histidine-tagged 14-3-3ζ or egg albumin proteins (Table 1, top).

His_6-ΔN222 was incubated with equimolar amounts of 14-3-3ζ or egg albumin and subjected to Ni^{2+} affinity chromatography. In the reaction mixture containing His_6-ΔN222 and 14-3-3ζ, essentially molar equivalents of each were reversibly bound to the affinity matrix (Table 1, bottom). Since 14-3-3ζ alone does not bind the resin (Table 1, top), these data indicate that 14-3-3ζ binds directly to His_6-ΔN222. In mixtures of egg albumin with His_6-ΔN222, egg albumin was eluted from the affinity resin in less than 0.1 molar equiv relative to His_6-ΔN222. To control for the possibility of nonspecific interaction of 14-3-3ζ with His_6-ΔN222, we examined the binding of 14-3-3ζ K49E, a charge reversal mutant that is 100-fold less potent than wild-type protein for the activation of ExoS (*14*). Only about 0.1 molar equiv of the K49E mutant was eluted from mixtures containing His_6-ΔN222, which indicated that the interaction of 14-3-3ζ with ExoS was specific.

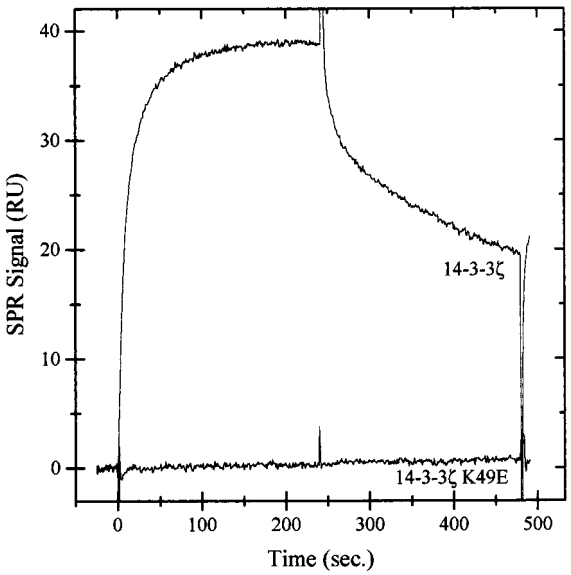

FIGURE 1: Typical ExoS binding sensorgrams for 14-3-3ζ wild type and K49E. 500 nM 14-3-3ζ wild type or K49E was passed over His_6-ΔN222 immobilized on a Ni^{2+}-NTA sensor chip (Biacore), and the mass at the surface of the chip was measured over time as a surface plasmon resonance response in a Biacore 2000 instrument. A parallel blank run of 14-3-3ζ wild type or K49E across a Ni^{2+}-NTA surface was subtracted from each curve. The flow of 14-3-3ζ started at time 0 and ended at time 240 s.

To provide an alternative measure of the interaction of 14-3-3ζ and ExoS, we performed surface plasmon resonance (SPR) studies using a Biacore 2000 instrument. His_6-ΔN222 was immobilized on a Ni^{2+} charged NTA coupled sensor chip, and 14-3-3ζ was flowed over the surface. Binding of 14-3-3ζ to ExoS caused an increase in mass at the surface of the chip which was reflected by an increase in SPR response over time. Nonspecific binding was low in this system, and it was removed from analysis by subtraction of a blank run of 14-3-3ζ over a Ni^{2+}-NTA surface. Typical blank subtracted sensorgrams are shown in Figure 1. Runs made at concentrations of 14-3-3ζ ranging from 20 to 500 nM were used to quantitatively determine the association (k_{on}) and dissociation (k_{off}) rate constants for the 14-3-3ζ/ExoS interaction. Assuming that 14-3-3ζ binds ExoS as a dimer, k_{on} was found to be $2.50 \times 10^5 \pm 1.5 \times 10^4$ M^{-1} s^{-1} (mean ± standard error; $n = 18$) and k_{off} was $1.79 \times 10^{-3} \pm 1.3 \times 10^{-4}$ s^{-1} ($n = 18$). The equilibrium dissociation constant for the interaction (K_D; $K_D = k_{off}/k_{on}$) was 7.2 ± 0.7 nM, indicating that His_6-ΔN222 and 14-3-3ζ bind directly and with high affinity. As in the affinity chromatography

Table 2. Effect of Phosphatase Treatment of ExoS on 14-3-3ζ Binding[a]

treatment	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)
none	$2.31 \times 10^5 \pm 2.0 \times 10^3$	$1.40 \times 10^{-3} \pm 1.3 \times 10^{-4}$	6.1 ± 0.6
mock	$2.97 \times 10^5 \pm 4.8 \times 10^3$	$2.02 \times 10^{-3} \pm 1.7 \times 10^{-4}$	6.8 ± 0.6
alkaline phosphatase	$3.24 \times 10^5 \pm 3.3 \times 10^3$	$1.87 \times 10^{-3} \pm 1.1 \times 10^{-4}$	5.8 ± 0.3

[a] His_6-ΔN222 was treated as indicated, then immobilized on an NTA sensor chip and tested for binding of 100 nM 14-3-3ζ by SPR. Rate constants were obtained using BIAevaluation 2.1 software as directed by the manufacturer. Values are mean ± standard error ($n = 3$).

experiments, 14-3-3ζ K49E was used to test the specificity of the interaction (Figure 1). 14-3-3ζ K49E did not detectably bind His_6-ΔN222.

Recombinant His_6-ΔN222 Is Not Phosphorylated. 14-3-3 is thought to be a phosphoserine binding protein, with preference for motifs similar to R-S-x-pS-x-P, where pS represents phosphorylated serine (*23*). The interaction of 14-3-3 with ExoS appears to be via a different mechanism, based on two observations. First, as demonstrated, 14-3-3 interacts with bacterially expressed ExoS, which would not be expected to be phosphorylated on serine since serine phosphorylation is not common in *E. coli*. Also, though full-length ExoS contains a sequence related to the putative 14-3-3 binding motif ([146]RSLSTA), this sequence is not present in His_6-ΔN222. To test the hypothesis that 14-3-3 interacts with ExoS in a phosphoserine-independent fashion, we used electrospray ionization mass spectrometry (ESI-MS) for determination of the amount of covalently bound phosphate in His_6-ΔN222. Tryptic peptides of His_6-ΔN222 were subjected to ESI-MS and subsequent analysis using the MassChrom software package for the presence of phosphorylated peptides. No phosphopeptide signals were found in the trypsin digests of 200 pmol of His_6-ΔN222 (data not shown). Under identical digestion and analysis conditions, 50 pmol of bovine β-casein yielded two phosphopeptides of mass 3123 and 2062 Da (data not shown). These masses match the predicted values for the known tryptic phosphopeptides of β-casein, residues 1−25 phosphorylated on serines 17, 18, 19, and 22 and residues 33−48 phosphorylated on serine 35, respectively. This critical positive control indicates that our preparation of His_6-ΔN222 is not likely phosphorylated. To provide additional support for this conclusion, we treated His_6-ΔN222 with calf intestinal alkaline phosphatase and tested for its binding to 14-3-3ζ by SPR. As shown in Table 2, alkaline phosphatase treatment did not cause a large change in the kinetic rate constants for the 14-3-3ζ/His_6-ΔN222 interaction compared to the mock treated control. These data are consistent with the model that 14-3-3 can bind to unphosphorylated ExoS.

Both Phosphorylated and Nonphosphorylated 14-3-3 Peptide Ligands Can Disrupt the ExoS/14-3-3 Interaction. The model that ExoS can bind 14-3-3 in a phosphorylation-independent manner was extended to determine whether the interaction site on 14-3-3 is distinct for ExoS and the more common phosphoserine-containing 14-3-3 ligands, such as Raf-1. To address this issue, we examined two 14-3-3 binding peptides for their effect on formation of the His_6-ΔN222/14-3-3ζ complex. The first peptide, R18, binds 14-3-3ζ in a nonphosphorylated state with a K_D of 89 nM (*22*). The second peptide was pS-Raf-259, which contains one of the R-S-x-pS-x-P motifs from Raf-1 and binds 14-3-3ζ with a K_D of 122 nM (*23*). These peptides were tested for their ability to block the 14-3-3ζ/His_6-ΔN222 interaction by equilibrating various concentrations with 14-3-3ζ and testing the resulting solution for binding to His_6-ΔN222. In this method, peptide binding causes a decrease in the free concentration of 14-3-3ζ, which is reflected by a decrease in 14-3-3 association with ExoS in the SPR assay. Both peptides inhibited the formation of the 14-3-3ζ/ExoS complex, and they did so with a similar concentration dependence (Figure 2). Competition for 14-3-3 binding between pS-Raf-259 and ExoS indicated that ExoS may bind 14-3-3 near 14-3-3's phosphoserine binding site, despite the lack of phosphoserine in ExoS.

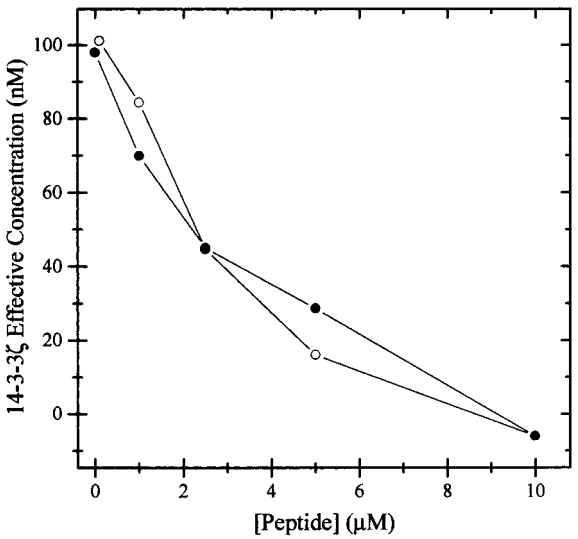

FIGURE 2: Both phosphorylated and nonphosphorylated 14-3-3 binding peptides inhibit the ExoS/14-3-3ζ interaction. Various concentrations of pS-Raf-259 (●) or R18 (○) were incubated with 100 nM 14-3-3ζ for 1 h, and the resulting mixtures were flowed over a His_6-ΔN222 surface in a Biacore 2000 instrument. The apparent concentration of 14-3-3ζ in the mixtures was estimated from the association phase of the sensorgram as described in the Methods section.

DISCUSSION

Among the family of bacterial ADP-ribosylating exotoxins, ExoS and cholera toxin/heat labile enterotoxin (CT/LT) are unique with respect to the requirement for a eukaryotic protein to express optimal ADP-ribosyltransferase activity (*33*). Members of the family of 14-3-3 proteins activate ExoS, while members of the family of ARF proteins activate CT/LT. The observation that *E. coli*-expressed recombinant 14-3-3ζ retains the ability to activate *E. coli*-expressed recombinant ExoS suggests that other *Pseudomonas* proteins are not involved in this activation process (*34*). Here we have used affinity chromatography and surface plasmon resonance techniques to demonstrate that the zeta isoform of 14-3-3 directly binds ExoS ΔN222, a C-terminal fragment of ExoS

that contains the 14-3-3-dependent catalytic domain. This binding is of high affinity, with a K_D of 7.2 nM. The interaction appears to be necessary for ExoS activation, since a 14-3-3 mutant that is 100-fold less potent at activating ExoS, 14-3-3ζ K49E, was unable to bind ExoS. We assume that this mutant does bind ExoS, but at a level below the detection limit of our assays, since 14-3-3ζ K49E is able to fully activate ExoS when it is present at very high concentrations (*14*).

14-3-3 ligands are generally thought to require phosphorylation on serine (*21*, *23*, *25*) before they can bind with high affinity. However, the form of ExoS used in these studies lacked 14-3-3 binding phosphoserine motifs or related sequences. Mass spectral analysis of ExoS detected no protein-bound phosphate, and treatment of ExoS with a nonspecific phosphatase had little effect on the interaction between 14-3-3 and ExoS. These data strongly argue against the possibility that the ExoS was phosphorylated during expression in *E. coli*. Given the low detection limit of the mass spectrometry analysis and the high stoichiometry of the 14-3-3/ExoS interaction (Table 1), we conclude that this interaction is not phosphoserine-dependent.

On the basis of our results supporting a non-phosphorylation-dependent interaction between 14-3-3 and ExoS, we questioned whether ExoS interacts with the same epitope on 14-3-3 as phosphorylated ligands do. It seems that this is the case, with the conserved amphipathic groove of 14-3-3 serving as the ExoS interaction site. The first piece of data to support this hypothesis is the lack of binding of 14-3-3ζ K49E to ExoS. This mutant has a charge reversal (positive to negative) in the charged face of the amphipathic groove. It has been shown that this mutation does not dramatically alter the three-dimensional structure of 14-3-3 as measured by partial proteolysis and circular dichroism, but that it does abolish binding of 14-3-3 to Raf-1 (*14*). Second, we demonstrated that pS-Raf-259, a phosphoserine-containing peptide that binds in the amphipathic groove of 14-3-3, inhibits the 14-3-3ζ/ExoS interaction in a potent fashion. Because a small peptide would not be expected to sterically hinder distant interactions, it is likely that pS-Raf-259 and ExoS share a common binding site on 14-3-3. The finding that a nonphosphorylated artificial ligand for 14-3-3, R18, was cocrystallized in a position similar to that of pS-Raf-259 (*22*) provides support for the possibility that natural nonphosphorylated ligands can interact in the amphipathic groove.

It is not surprising that 14-3-3 can directly bind nonphosphorylated ExoS. Structural requirements for binding to 14-3-3 can be satisfied by phosphoserine motifs, but also by an amphipathic peptide of appropriate properties. In the cocrystal structure of R18 with 14-3-3ζ (*22*) five peptide residues were resolved, consisting of the sequence WL**D**L**E**. The aspartate and glutamate side chains created a negative charge density in a fashion similar to that of phosphoserine (Figure 3), and the tryptophan and leucine residues made hydrophobic contacts on the opposite side of the amphipathic groove. There is a homologous sequence in ExoS ΔN222, ^{245}FGA**D**A**E**, that may be a component of the 14-3-3 binding epitope of ExoS. This motif could play a role in the 14-3-3/ExoS interaction by binding in the amphipathic groove in a fashion similar to R18.

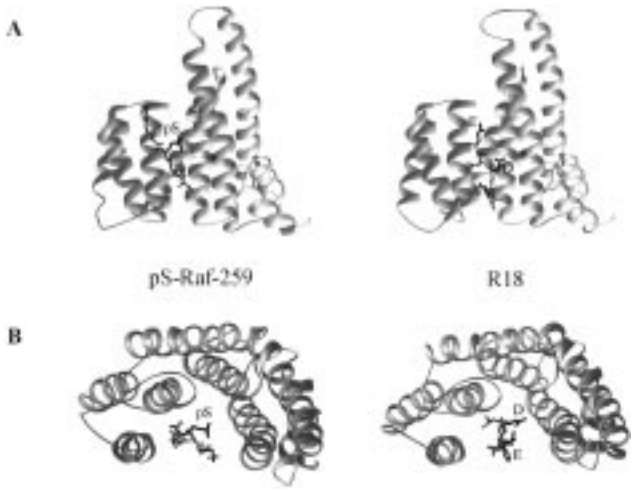

FIGURE 3: Structural model of 14-3-3ζ cocrystallized with pS-Raf-259 and R18 peptides. (A) A single monomer from each of the 14-3-3ζ cocrystal structures (22) is shown. Both phosphoserine (pS-Raf-259) and non-phosphoserine-containing (R18) peptides bind 14-3-3ζ in its amphipathic groove. Aspartate and glutamate residues from R18 (labeled D and E) are positioned to create a negative charge density similar to that of the phosphoserine of pS-Raf-259 (labeled pS). This negative charge interacts with several basic residues of 14-3-3ζ, including lysine 49 (Cα approximate position marked with *). (B) The same structures as in A, rotated 90° to show the amphipathic groove. Labeling is as in A, except that lysine 49 is not marked. This figure was prepared using the program Ribbons (35).

In summary, we have demonstrated that 14-3-3ζ can directly bind to ExoS and that this interaction involves the conserved amphipathic groove of 14-3-3. This has important implications because ExoS is not phosphorylated on serine. In addition, our data have pointed to a candidate 14-3-3 binding site on ExoS. Our findings raise the possibility that 14-3-3 may bind both phosphorylated and nonphosphorylated partners in cells. The association of 14-3-3 with nonphosphorylated ligands may create constitutive interactions. It is also possible that this type of binding is regulated in a kinase-independent manner, that is, by protein−protein interaction-induced conformational changes.

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