Residues of 14-3-3ζ Required for Activation of Exoenzyme S of *Pseudomonas aeruginosa*†

Lixin Zhang,[‡,§] Haining Wang,[∥] Shane C. Masters,[⊥] Bingcheng Wang,[▽] Joseph T. Barbieri,[○] and Haian Fu*[,‡]

Department of Pharmacology and Graduate Programs in Cell and Developmental Biology and Molecular Therapeutics and Toxicology, Emory University, Atlanta, Georgia 30322, Rammelkamp Center for Research, Case Western Reserve University, Cleveland, Ohio 44109, and Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Received May 4, 1999; Revised Manuscript Received July 13, 1999

ABSTRACT: Exoenzyme S (ExoS) is a mono-ADP-ribosyltransferase secreted by the opportunistic pathogen *Pseudomonas aeruginosa*. ExoS requires a eukaryotic factor, the 14-3-3 protein, for enzymatic activity. Here, two aspects of the activation of the ADP-ribosyltransferase activity of ExoS by 14-3-3 proteins are examined. Initial studies showed that several isoforms of 14-3-3, including β, ζ, η, σ, and τ, activated ExoS with similar efficiency. This implicates a conserved structure in 14-3-3 that contributes to the interaction between 14-3-3 and ExoS. One candidate structure is the conserved amphipathic groove that mediates the 14-3-3/Raf-1 interaction. The next series of experiments examined the role of individual amino acids of the amphipathic groove of 14-3-3ζ in ExoS activation and showed that ExoS activation required the basic residues lining the amphipathic groove of 14-3-3ζ without extensive involvement of the hydrophobic residues. Strikingly, mutations of Val-176 of 14-3-3ζ that disrupted its interaction with Raf-1 did not affect the binding and activation of ExoS by 14-3-3. Thus, ExoS selectively employs residues in the Raf-binding groove for its association with 14-3-3 proteins.

Bacterial ADP-ribosylating toxins catalyze the cleavage of NAD^+ at the glycosidic bond and covalently attach the ADP-ribose (ADPR)[1] moiety to a eukaryotic target protein (*1*, *2*). Many of these targeted proteins are nucleotide-binding proteins such as the heterotrimeric G proteins, which play critical roles in regulating diverse cellular functions (*3*). ADP-ribosylation of these nucleotide-binding proteins and the subsequent alteration of their functions are responsible for the pathological manifestation of the toxins. Among the ADP-ribosylating exotoxins, cholera toxin and ExoS of *Pseudomonas aeruginosa* are unique in that they require host factors for activation, suggesting the contribution of eukaryotic host proteins in bacterial pathogenesis (*4*−*6*). Elucidating how host proteins enhance the enzymatic activity of bacterial toxins may lead to a better understanding of the mechanism of bacterial infection and thereby provide novel strategies for therapeutic intervention.

ExoS is secreted by the opportunistic pathogen *P. aeruginosa* (*7*) and delivered into eukaryotic cells by the type III secretion apparatus (*8*). ExoS appears to contribute to the virulence of *P. aeruginosa* (*9*), although the role of ExoS in *P. aeruginosa* pathogenesis remains unclear. Recently, intracellular expression of ExoS has been shown to be toxic to cultured cells (*10*, *11*), which may partially explain its role in infections by *P. aeruginosa*. In vitro, ExoS ADP-ribosylates a number of eukaryotic substrates, including vimentin (*12*), IgG3 (*13*), and the small GTP-binding proteins Ras and Rab (*14*). ADP-ribosylation of Ras by ExoS has been demonstrated in eukaryotic cells (*15*). Covalent modification of multiple cellular constituents may account for its cytotoxic effects. Interestingly, the enzymatic activity of ExoS requires the presence of a eukaryotic factor identified as 14-3-3ζ protein (*16*).

14-3-3 is a family of dimeric molecules with seven isoforms known in mammalian cells (β, γ, ϵ, ζ, η, σ, and τ; *17*). 14-3-3 proteins were originally identified as abundant brain proteins and later found to be widespread in eukaryotic tissues and ubiquitous in eukaryotic organisms. Diverse biological activities have been attributed to members of the 14-3-3 family, including regulation of cell cycle progression (*18*, *19*), apoptosis (*20*), viral transformation (*21*), and bacterial pathogenesis (*16*). The ability of 14-3-3 proteins to interact with a wide range of cell regulatory proteins and virulence factors may account for these important functions.

† This work was supported in part by NIH Grants GM53165 to H.F., GM08602 to S.C.M., and DK54178 to B.W. H.F. is a recipient of the Burroughs Wellcome Fund New Investigator Award and the PhRMA Faculty Development Award.

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

‡ Department of Pharmacology, Emory University.

§ Present address: Microbia Inc., 840 Memorial Dr., Cambridge, MA 02139.

∥ Graduate Program in Cell and Developmental Biology, Emory University.

⊥ Graduate Program in Molecular Therapeutics and Toxicology, Emory University.

▽ Case Western Reserve University.

○ Medical College of Wisconsin.

[1] Abbreviations: ADPR, ADP-ribose; ExoS, exoenzyme S; GST, glutathione *S*-transferase; SBTI, soybean trypsin inhibitor; WT, wild type.

10.1021/bi991019l CCC: $18.00
Published on Web 08/26/1999

Thus, identification of structural determinants required for 14-3-3/ligand interaction is critical for dissecting the role of 14-3-3 in cellular regulation and microbial pathogenesis.

Many 14-3-3-associated proteins bind in a phosphorylation-dependent manner (*22*, *23*). Muslin et al. defined a phosphoserine-containing motif, RSXpSXP as a prototype, which serves as a 14-3-3 recognition site (*24*, *25*; X represents any amino acid, while pS stands for phosphorylated serine). It appears that this phosphoserine motif provides a general mechanism for 14-3-3/ligand interactions. For example, mutations of Raf-1 at Ser-259 of the RSXpS^{259}XP motif disrupt the Raf-1/14-3-3 interaction (*23*, *24*), while mutation at Ser-136 in the 14-3-3 binding motif of Bad, a proapoptotic protein, abolishes the Bad/14-3-3 interaction and Akt-induced cell survival (*20*, *26*). To accommodate the binding of phosphorylated ligands, 14-3-3 proteins employ a conserved groove formed by surface residues from four of its α-helices, a model (*27*) that has been supported by both structural and mutational studies (*25*, *28*−*31*). The binding groove of 14-3-3ζ exhibits an amphipathic nature with a cluster of positively charged residues on one side of the groove (K49, R56, K120, and R127) and several hydrophobic residues on the other side including V176, L216, L220, and L227 (*27*). Mutations that alter the amphipathic nature of the groove decrease the affinity of 14-3-3 to Raf-1 (*29*, *30*). Recent cocrystallization analysis shows that the phosphoserine peptides derived from Raf-1 and the middle T antigen of polyomavirus are indeed located in this amphipathic groove (*25*, *28*). Thus, it is possible that recognition of phosphoserine motifs in target proteins by the conserved binding groove of 14-3-3 determines specific 14-3-3/ligand interaction.

Unlike Raf-1 and Bad, ExoS is not known to be phosphorylated (*32*). To address the mechanism of ExoS activation by 14-3-3 proteins, we tested the requirement of residues in the conserved ligand binding groove of 14-3-3ζ for ExoS activation. Using a defined ADP-ribosylation assay, we have found that the basic cluster in the binding groove of 14-3-3ζ, including Lys-49, Arg-56, Lys-120, and Arg-127, is pivotal for activation of ExoS, while residues on the hydrophobic surface of the groove are dispensable. Thus ExoS selectively utilizes a subset of residues in the conserved binding groove of 14-3-3ζ for its activation. Selective sampling of residues in the amphipathic groove of 14-3-3 by different 14-3-3-binding proteins may contribute to ligand selectivity.

MATERIALS AND METHODS

Strains and Plasmids. *Escherichia coli* strains were grown at 37 °C in LB medium. Strain XL1-Blue (Stratagene, La Jolla, CA) was used for propagation of plasmids, strain MutS was used to propagate mutagenized plasmids for site-directed mutagenesis, and strain BL21(DE3) was used for protein expression. pHAF625 (*29*), a pUC19-based plasmid carrying the 14-3-3ζ gene, was used for mutagenesis. For protein expression in *E. coli*, cDNAs of 14-3-3 isoforms and mutated derivatives were subcloned into the pET-15b expression vector (Novagen, Madison, WI) as described[2] (*16*, *29*).

Protein Expression and Purification. The expression of hexahistidine-tagged fusion proteins was induced with IPTG as previously described (*29*). Briefly, cells were sonicated on ice and then subjected to centrifugation (13000*g*, 10 min). The supernatants were the source of hexahistidine-tagged 14-3-3 proteins and were directly used for solid-phase binding assays as described below. For protein purification, the supernatants were subjected to Ni^{2+}-chelating chromatography on the Pharmacia FPLC system (*29*). ExoS was purified essentially as described (*33*).

ExoS Activation Assay. The NAD^+:SBTI ADP-ribosyltransferase assay measures the rate of incorporation of the [^{32}P]ADPR moiety of NAD^+ into trichloroacetic acid-precipitable material (SBTI). ADP-ribosylation of SBTI (*6*) was performed as previously described (*29*, *33*). The reaction mixtures contained (in a final volume of 25 μL) 0.2 M sodium acetate (pH 6.0), 20 nM purified ExoS, 30 μM SBTI, 30 μM [*adenylate*-^{32}P-phosphate]NAD^+, 1.5 μM bovine serum albumin, and varying concentrations of 14-3-3 or mutated derivatives. Activities were obtained from at least three separate experiments, each performed in duplicate. ExoS activation data were fit to the following equation by Sigma Plot (SPSS, Chicago, IL): $v = V_{max}[A]/(EC_{50} + [A])$, where v is the observed enzyme activity expressed as picomoles of ADPR incorporated per minute per picomole of ExoS, V_{max} is the maximal activation, [A] is the 14-3-3 concentration, and EC_{50} is the concentration of 14-3-3 proteins giving half-maximal activation. In the peptide competition assay, purified 14-3-3ζ protein was preincubated with increasing concentrations of GST-R18 or GST alone at 25 °C for 30 min. The reactions were started by adding aliquots of the pretreated 14-3-3ζ (20 nM final concentration) and were carried out as described above.

Site-Directed Mutagenesis. Site-directed mutagenesis of the 14-3-3ζ gene was performed with the unique-site-elimination method, using double-stranded plasmid as a starting template essentially as described (*29*). The mutagenic primers used are listed below (mutations generated are underlined, and the restriction sites introduced or abolished for screening purposes are in lowercase): (1) 5′-GTCTTCTAtctagAATGAAA-3′ (Lys-120 to Glu, *Xba*I site introuced); (2) 5′-GGAGACTACTAcgAGTACTTggCTGAGG-3′ (Arg-127 to Glu, *Bsl*I site abolished); (3) 5′-CTTCTCTGCGTTCTATTATGagatctTGAACTCC-3′ (Val-176 to Ala, *Bgl*II site introduced); (4) 5′-AACTTCTCTAAGTTCTATTATGagatctTGAACTCC-3′ (Val-176 to Lys, *Bgl*II site introduced). The construction of other 14-3-3ζ mutants has been described previously (*29*, *30*).

Solid-Phase Binding Experiments. Ni^{2+}-chelated His-Bind beads (Novagen, Madison, WI) were incubated with *E. coli* crude extracts containing hexahistidine-tagged WT or mutant 14-3-3ζ proteins to generate beads coated with 14-3-3, which were used in subsequent binding assays. Radiolabeled ExoS and Raf-1 were generated by the TNT in vitro transcription/translation system (Promega, Madison, WI), according to the manufacturer's specifications. For the binding assay, 14-3-3 coated beads (∼5 μg of protein per reaction) were incubated with 5 μL of TNT reaction mix containing [^{35}S]methionine-labeled ExoS or Raf-1. The 14-3-3ζ coated beads with associated proteins were washed three times with Nonidet P-40 buffer (1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, and 40 mM Tris-HCl, pH 8.0). The protein complexes formed in the binding assay were eluted from the beads by boiling in 10 μL of SDS sample buffer and resolved by

[2] R. R. Subramanian, S. C. Masters, and H. Fu, unpublished data.

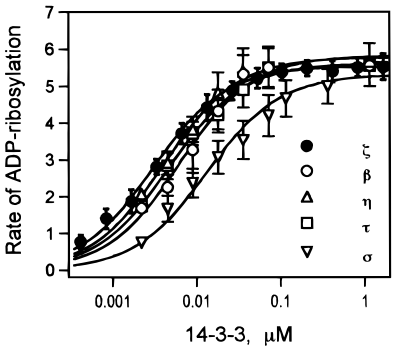

FIGURE 1: Activation of ExoS by isoforms of 14-3-3 proteins. Isoforms of 14-3-3 proteins were expressed in *E. coli* and purified to near homogeneity by sequential nickel-chelate and gel-filtration chromatography (*29*). The indicated amounts of 14-3-3 proteins were assayed for activation of ExoS activity as described under Materials and Methods. The reactions were carried out for 30 min at 25 °C. The rate of ADPR incorporation into the substrate SBTI is expressed as picomoles of ADP-ribose incorporated per minute per picomole of ExoS. The specific incorporation of [^{32}P]ADP-ribose into the substrate SBTI was confirmed by an SDS−PAGE-based assay (data not shown). Error bars represent mean ± standard error ($n = 6$).

SDS−PAGE. The presence of radiolabeled ExoS or Raf-1 protein in the 14-3-3 complex was visualized by use of a PhosphorImager (Molecular Dynamics, Inc.).

SDS−PAGE and Protein Assay. SDS−PAGE was performed essentially as described by Laemmli (*34*). Protein concentration was determined by use of a protein assay kit (Bio-Rad, Hercules, CA) based on the method of Bradford (*35*) with BSA as a standard.

RESULTS

ADP-Ribosyltransferase Activity of ExoS Depends on 14-3-3 Proteins. To express ADP-ribosyltransferase activity, ExoS requires the presence of a eukaryotic cofactor, identified as 14-3-3ζ (*16*). To determine the structural requirements for ExoS activation by 14-3-3ζ, we tested whether different mammalian isoforms of 14-3-3 have conserved this function. As previously reported, ADP-ribosyltransferase activity was not detected when ExoS was incubated with its substrates, NAD^+ and SBTI, but without 14-3-3 (*6*, *16*). Addition of 14-3-3ζ to this reaction activated ExoS to catalyze ADP-ribosylation of SBTI, as evidenced by the increased radioactive counts in the trichloroacetic acid-precipitable fraction (Figure 1) and the specific radiolabelling of SBTI (data not shown). The cofactor activity of the 14-3-3 proteins for ExoS is not restricted to a specific isotype, since purified ζ, *β*, *η*, *σ*, and *τ* isoforms all activated the ADP-ribosyltransferase activity of ExoS. The EC_{50}s of the ζ, *β*, *η*, *σ*, and *τ* isoforms were 3.1, 4.7, 6.3, 13.2, and 4.1 nM, respectively. All of the tested 14-3-3 proteins gave similar V_{max} values, indicating that they are equally efficacious. This indicates that the cofactor activity for ExoS is a general property of the 14-3-3 protein family, which suggests that a conserved structure within 14-3-3 proteins mediates the ExoS/14-3-3 interaction.

The Conserved Amphipathic Groove of 14-3-3ζ Is Involved in the Activation of ExoS. Recently a primary ligand binding site involving an amphipathic groove of 14-3-3ζ was identified by a combination of structural and genetic analysis (*25*, *27*−*31*, *36*). Surface residues that make up this amphipathic groove are conserved among all the 14-3-3 isoforms.

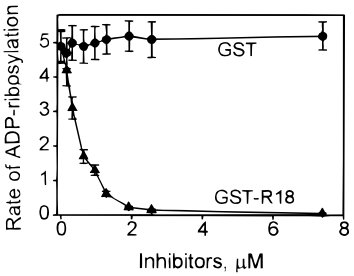

FIGURE 2: Inhibition of 14-3-3ζ-dependent activation of ExoS by the R18 peptide. Different concentrations of GST−R18 or GST proteins were incubated with 14-3-3ζ prior to addition to the ADP-ribosylation reaction mix. Experiments were performed as described in the caption to Figure 1 with a constant level of 14-3-3ζ (20 nM). Error bars represent mean ± standard error ($n = 6$).

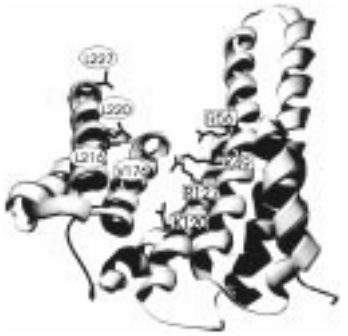

FIGURE 3: Atomic model of the 14-3-3ζ monomer. Residues lining the conserved amphipathic groove (*27*) and analyzed in this study are shown. This figure was generated by use of the programs Swiss-PdbViewer version 3.1 (*42*) and POV-Ray for Windows version 3.1.

To test whether the amphipathic groove is also involved in activating ExoS, we used a synthetic peptide ligand, R18, as a molecular probe. The R18 peptide was isolated from a phage display library for its high affinity for 14-3-3 (*43*). In the cocrystal structure of 14-3-3ζ in complex with R18, the core sequence of R18 (WLDLE) binds to the 14-3-3ζ amphipathic groove (*28*). If the amphipathic groove of 14-3-3ζ is important for the activation of the ADP-ribosyltransferase activity of ExoS, blockage of this groove with R18 may inhibit 14-3-3-dependent ExoS activation. As shown in Figure 2, preincubation of 14-3-3ζ with GST−R18 reduced the ability of 14-3-3ζ to activate ExoS, while preincubation with GST alone showed no inhibitory effect. The inhibitory activity of GST−R18 was dose-dependent with an IC_{50} of about 200 nM. Similar results were obtained with chemically synthesized R18 peptide (data not shown). These data are consistent with the model that the conserved amphipathic groove of 14-3-3ζ is involved in the activation of ExoS.

Mutations in the Amphipathic Groove of 14-3-3ζ Differentially Affect Its Ability To Activate ExoS. Crystallographic studies of 14-3-3ζ predicted the participation of a number of residues in ligand binding (Figure 3; *25*, *27*, *28*). A cluster of positively charged residues in the amphipathic groove appear to be the contact sites for the phosphoserine moiety of phosphorylated ligands (*25*, *27*). On the adjacent face of the groove, a patch of hydrophobic residues, including Val-176, Leu-216, Leu-220, and Leu-227, also appear to contribute to ligand binding (*27*, *30*). The involvement of the two faces of the amphipathic groove of 14-3-3ζ in ExoS activation was tested by measuring the effect of mutating

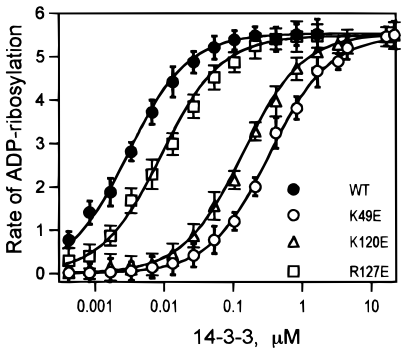

FIGURE 4: Comparison of WT and charge-reversal mutants of 14-3-3ζ for activation of ExoS ADP-ribosyltransferase. Mutant proteins were prepared and the effect of mutations K49E, K120E, and R127E of 14-3-3ζ on its activation of ExoS was analyzed as described in the caption to Figure 1. Error bars represent mean ± standard error ($n = 6$).

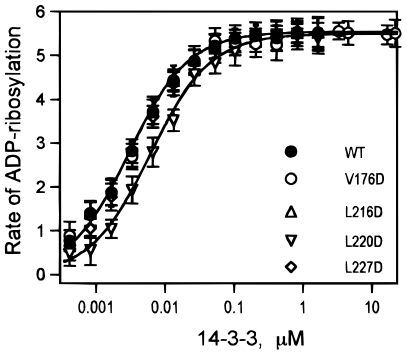

FIGURE 5: Effect of mutations V176D, L216D, L220D, and L227D of 14-3-3ζ on its activation of ExoS. Experiments were performed as described in the caption to Figure 1. Error bars represent mean ± standard error ($n = 6$).

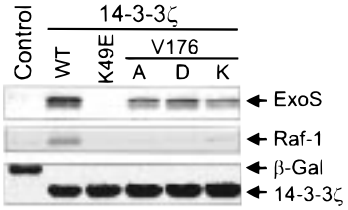

FIGURE 6: Comparison of in vitro binding of ExoS and Raf-1 to WT and mutant 14-3-3ζ proteins. ExoS and Raf-1 proteins were generated in vitro by a rabbit reticulocyte lysate transcription and translation system in the presence of [^{35}S]methionine (*29*). Equal portions of the labeled ExoS or Raf-1 protein were incubated for 1 h at 4 °C under constant rotation with hexahistidine-tagged WT or mutated 14-3-3ζ proteins or a control protein, *β*-galactosidase, bound to His-Bind beads (Novagen). The beads with immobilized WT or mutant 14-3-3ζ protein complexes were washed extensively as described under Materials and Methods. The bound proteins were eluted with SDS sample buffer, resolved by SDS−PAGE (12.5%), and visualized by use of a PhosphorImager (top and middle panels) or by Coomassie Blue staining (bottom panel).

these residues on the activation of ExoS ADP-ribosyltransferase activity.

Previous studies indicated that Lys-49 and Arg-56 of 14-3-3ζ were important for Raf-1 binding, as well as for activating ExoS (*29*), suggesting that the basic cluster is required for ExoS activation. We extended this analysis to test the involvement of Lys-120 and Arg-127 in ExoS activation. Lys-120 and Arg-127 of 14-3-3ζ were individually changed to Glu. Mutated proteins were compared to WT 14-3-3ζ protein and the point mutant 14-3-3ζ^{K49E} for the ability to activate ExoS (Figure 4). Consistent with earlier studies, the charge-reversal mutation K49E drastically increased the EC_{50} of 14-3-3ζ for ExoS by about 100-fold relative to WT 14-3-3ζ. Similarly, the charge-reversal mutations K120E and R127E of 14-3-3ζ increased the EC_{50} of 14-3-3ζ by 46-fold and 4-fold, respectively. None of these mutations had any significant effect on the V_{max} of activated ExoS, suggesting that charged residues in the amphipathic groove of 14-3-3ζ are not directly involved in ExoS catalysis. Also, the reduced activity of mutant 14-3-3 proteins is not a result of gross structural changes because they can form dimers and exhibited similar proteolytic patterns and far-UV circular dichroism spectra as that of WT (data not shown). Together, these results demonstrate that residues Lys-120 and Arg-127 in the amphipathic groove of 14-3-3ζ, like Lys-49 and Arg-56, are part of a structural determinant critical for activation of ExoS.

It has been shown that residues in the hydrophobic face of the amphipathic groove of 14-3-3ζ (V176, L216, L220, and L227) are involved in its association with Raf-1 to various extents. In the cocrystal structure of 14-3-3ζ with a phosphorylated Raf-259 peptide (*28*), the side chain of Val-176 is about 5 Å away from the phosphate group of the peptide. Thus, the side chain of Val-176 may directly contact the side chains of the Raf-1 peptide. In support of this model, 14-3-3ζ^{V176D} did not bind to Raf-1 (*30*). To examine whether hydrophobic residues within the amphipathic groove of 14-3-3ζ that are involved in Raf-1 binding are also required for ExoS activation, we introduced a negatively charged Asp into the hydrophobic face of the groove, generating the 14-3-3ζ mutants V176D, L216D, L220D, and L227D (*30*). Mutated proteins were assayed relative to WT 14-3-3ζ for their ability to activate the ADP-ribosyltransferase activity of ExoS (Figure 5). Although a single point mutation, V176D, abolished the binding of 14-3-3 to Raf-1, this mutation did not have a detectable effect on the ability of 14-3-3ζ to activate the ADP-ribosyltransferase activity of ExoS. L220D exhibited a slight effect on the EC_{50} of 14-3-3ζ (about 2-fold increase), while L216D and L227D showed no obvious effect. These mutated proteins did not affect the V_{max} of ExoS. Thus, the hydrophobic residues within the amphipathic groove of 14-3-3ζ do not appear to be essential for 14-3-3ζ to activate ExoS.

Association of ExoS with 14-3-3ζ Is Unaffected by Several Mutations at Val-176. We have shown that ExoS can directly bind 14-3-3ζ (*32*). To confirm that residues pivotal for Raf-1 binding are dispensable for ExoS interaction, we directly tested the effect of mutations at Val-176 of 14-3-3ζ on binding to ExoS. Val-176 was mutated to Ala and Lys, in addition to Asp, which was prepared earlier. As shown in Figure 6, these replacements did not substitute for Val-176 with respect to Raf-1 binding. This is consistent with the cocrystal structure, which showed the side chain of Val-176 to be directly involved in contacting the side chains of the Raf-1 peptide (*28*). In contrast, changing Val-176 to Asp, Lys, or Ala did not significantly affect the ability of 14-3-3ζ to bind ExoS. In agreement with these results, mutations at V176 of 14-3-3ζ did not alter the EC_{50} or V_{max} for ExoS activation (data not shown). In general, we found that decreased 14-3-3 binding is correlated with the ability of 14-3-3 to activate ExoS. For example, both 14-3-3ζ^{K49E} and

14-3-3ζ^{K120E} proteins exhibited drastically increased EC_{50} for ExoS (Figure 4) as well as diminished binding to ExoS (Figure 6; data not shown). Together, these data indicate that 14-3-3 is an allosteric activator of ExoS and that 14-3-3 binds both ExoS and Raf-1 with its amphipathic groove but with different subsets of residues.

DISCUSSION

We showed here that different isoforms of 14-3-3 proteins can activate ExoS with similar efficiency, suggesting a structure conserved among 14-3-3 proteins is involved in 14-3-3/ExoS interaction. This observation is consistent with a model where the amphipathic groove formed by surface residues conserved throughout the 14-3-3 family represents the primary ligand-binding site. In addition to Lys-49 and Arg-56, this study identified Lys-120 and Arg-127 in the amphipathic groove of 14-3-3ζ as important structural determinants for activation of ExoS. Our experiments also demonstrated that the hydrophobic residue Val-176 of 14-3-3ζ, which was critical for Raf-1 binding, was dispensable for ExoS association. Thus, while ExoS and Raf-1 share an overlapping structural determinant on 14-3-3ζ, the ExoS/14-3-3 interaction does not involve hydrophobic residues in the amphipathic groove.

Our data indicate that 14-3-3ζ residues Lys-49, Arg-56, Lys-120, and Arg-127 are required for activation of ExoS (Figure 4; *29*). These positively charged residues are solvent-accessible and available in principle for ligand interaction (*25*, *27*, *28*). These four residues are absolutely conserved among all the mammalian 14-3-3 isoforms (*28*), consistent with the ability of the various isoforms of 14-3-3 to activate ExoS (Figure 1). In the cocrystal structure of 14-3-3ζ and R18, residues of R18 are located next to side chains of the positively charged cluster, including Lys-49, Arg-56, Lys-120, and Arg-127 (*28*). Overlapping interaction of R18 and ExoS with the same set of residues of 14-3-3ζ may account for the inhibitory effect of R18 on ExoS activation by the 14-3-3 proteins (Figure 2). Interestingly, this same set of 14-3-3ζ residues are also determinants for contacting phosphoserine in phosphorylated ligands (*25*, *28*). In the cocrystal structure of 14-3-3ζ in complex with a phosphoserine-containing peptide derived from polyomavirus middle T antigen, the side chains of Lys-49, Arg-56, and Arg-127 form salt bridges to the phosphate group of the phosphoserine-containing peptide, while part of the Lys-49 and Lys-120 side chains are also involved in contacting the side chains of residues adjacent to the phosphoserine (*25*). It is possible that a structurally similar determinant in ExoS mimics the phosphoserine motif that mediates the 14-3-3 interaction. The structure of R18 may provide a clue for ExoS. In the unphosphorylated R18 peptide, two negatively charged residues, Asp and Glu, with appropriate spacing as shown in the WL**D**L**E** structure, may mimic the phosphoserine group (*28*). This peptide assumes an extended, amphipathic structure with hydrophobic residues contacting the hydrophobic face of the groove, while negatively charged residues contact the positively charged cluster. We have identified a homologous motif in ExoS, ^{245}FGA**D**A**E**, that may mediate the ExoS/14-3-3 interaction in a fashion similar to R18 (*32*). However, it is also possible that ExoS may use a modified motif for its interaction with 14-3-3 because the hydrophobic face of the ligand binding groove, including Val-176, is not essential for interaction of 14-3-3ζ with ExoS (Figure 6). Thus, ExoS may contact a different set of hydrophobic residues in the amphipathic groove or utilize a distinct motif, involving charged residues, for its interaction with 14-3-3.

ExoS production from *P. aeruginosa* has been associated with increased virulence in burn wounds and chronic lung infections (*37*−*39*) and with increased cell injury (*40*, *41*). Recent understanding of the type III delivery system of *P. aeruginosa* for ExoS has led to the observation that ExoS can inhibit cell proliferation and induce toxicity in eukaryotic cells (*10*, *11*). The antiproliferation property is correlated with the ADP-ribosyltransferase activity of ExoS to modify endogenous substrates such as Ras in host cells (*15*). Because the catalytic activity of ExoS requires its interaction with the host protein 14-3-3, inhibition of the 14-3-3/ExoS interaction may impair the pathological effect of ExoS. Our study has revealed a specific 14-3-3 site for ExoS interaction, which includes a cluster of basic residues, Lys-49, Arg-56, Lys-120, and Arg-127. Targeting this site with 14-3-3 antagonists, such as R18 or small molecules, may inhibit ExoS binding, thus attenuating the ExoS effect during *Pseudomonas* infections. An alternative approach would be to design molecules that mimic the 14-3-3 binding site on ExoS, which may decrease potential nonspecific side effects, since the target is the cytotoxin rather than a host protein.

This study showed that ExoS and the kinase Raf-1 share a subset of residues in the ligand binding groove of 14-3-3ζ. It is possible that ExoS can compete with other endogenous ligands for 14-3-3 binding. Although the enzymatic activity of ExoS appears to be its primary pathogenic determinant, the ability of ExoS to compete with endogenous proteins for 14-3-3 binding could lead to the dissociation of 14-3-3 from its physiological ligands. Thus, the binding of ExoS to 14-3-3 may have dual effects in the cell that lead to (i) the ADP-ribosylation of multiple cellular substrates and (ii) the disruption of 14-3-3-mediated functions, such as promoting Raf-1-mediated cell proliferation. It is also worth noting that ExoS can be activated by different isoforms of 14-3-3 proteins. Since isoforms of 14-3-3 are expressed in different tissues, ubiquitous activation of ExoS by 14-3-3 isoforms allows enzymatic activation of ExoS in many tissues.

In summary, the unphosphorylated ExoS and phosphoserine-containing ligands, such as Raf-1, use the same group of positively charged residues in 14-3-3ζ as contact sites. Unlike phosphorylated ligands, ExoS does not require hydrophobic residues in the conserved binding groove for this interaction. Future studies may lead to designing specific inhibitors that block ExoS activation by 14-3-3, which could have therapeutic potential against *Pseudomonas* infection.

ACKNOWLEDGMENT

We thank Keith Wilkinson, Dale Edmonson, Romesh Subramanian, Jing Chen, and Hongzhu Yang for helpful discussions.

REFERENCES

1. Collier, R. J. (1975) *Bacteriol. Rev. 39*, 54−85.
2. Middlebrook, J. L., and Dorland, R. B. (1984) *Microbiol. Rev. 48*, 199−221.
3. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature 349*, 117−27.

4. Kahn, R. A., and Gilman, A. G. (1984) *J. Biol. Chem. 259*, 6228−34.
5. Gill, D. M., and Coburn, J. (1987) *Biochemistry 26*, 6364−71.
6. Coburn, J., Kane, A. V., Feig, L., and Gill, D. M. (1991) *J. Biol. Chem. 266*, 6438−46.
7. Iglewski, B. H., Sadoff, J., Bjorn, M. J., and Maxwell, E. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3211−5.
8. Yahr, T. L., Goranson, J., and Frank, D. W. (1996) *Mol. Microbiol. 22*, 991−1003.
9. Coburn, J. (1992) *Curr. Top. Microbiol. Immunol. 175*, 133−43.
10. Olson, J. C., McGuffie, E. M., and Frank, D. W. (1997) *Infect. Immun. 65*, 248−56.
11. Frithz-Lindsten, E., Du, Y., Rosqvist, R., and Forsberg, A. (1997) *Mol. Microbiol. 25*, 1125−39.
12. Coburn, J., Dillon, S. T., Iglewski, B. H., and Gill, D. M. (1989) *Infect. Immun. 57*, 996−8.
13. Knight, D. A., and Barbieri, J. T. (1997) *Infect. Immun. 65*, 3304−9.
14. Coburn, J., and Gill, D. M. (1991) *Infect. Immun. 59*, 4259−62.
15. McGuffie, E. M., Frank, D. W., Vincent, T. S., and Olson, J. C. (1998) *Infect. Immun. 66*, 2607−13.
16. Fu, H., Coburn, J., and Collier, R. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2320−4.
17. Aitken, A. (1996) *Trends Cell Biol. 6*, 341−347.
18. Ford, J. C., al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J., and Carr, A. M. (1994) *Science 265*, 533−5.
19. Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997) *Science 277*, 1501−5.
20. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell 87*, 619−28.
21. Pallas, D. C., Fu, H., Haehnel, L. C., Weller, W., Collier, R. J., and Roberts, T. M. (1994) *Science 265*, 535−7.
22. Furukawa, Y., Ikuta, N., Omata, S., Yamauchi, T., Isobe, T., and Ichimura, T. (1993) *Biochem. Biophys. Res. Commun. 194*, 144−9.
23. Michaud, N. R., Fabian, J. R., Mathes, K. D., and Morrison, D. K. (1995) *Mol. Cell. Biol. 15*, 3390−7.
24. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell 84*, 889−97.
25. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) *Cell 91*, 961−71.
26. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell 91*, 231−41.
27. Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) *Nature 376*, 191−4.
28. Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H., and Liddington, R. C. (1998) *J. Biol. Chem. 273*, 16305−10.
29. Zhang, L., Wang, H., Liu, D., Liddington, R., and Fu, H. (1997) *J. Biol. Chem. 272*, 13717−24.
30. Wang, H., Zhang, L., Liddington, R., and Fu, H. (1998) *J. Biol. Chem. 273*, 16297−304.
31. Thorson, J. A., Yu, L. W. K., Hsu, A. L., Shih, N. Y., Graves, P. R., Tanner, J. W., Allen, P. M., Piwnica-Worms, H., and Shaw, A. S. (1998) *Mol. Cell. Biol. 18*, 5229−38.
32. Masters, S. C., Pederson, K. J., Zhang, L., Barbieri, J. T., and Fu, H. (1999) *Biochemistry 38* 5216−5221.
33. Kulich, S. M., Frank, D. W., and Barbieri, J. T. (1993) *Infect. Immun. 61*, 307−13.
34. Laemmli, U. K. (1970) *Nature 227*, 680−5.
35. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−54.
36. Xiao, B., Smerdon, S. J., Jones, D. H., Dodson, G. G., Soneji, Y., Aitken, A., and Gamblin, S. J. (1995) *Nature 376*, 188−91.
37. Bjorn, M. J., Pavlovskis, O. R., Thompson, M. R., and Iglewski, B. H. (1979) *Infect. Immun. 24*, 837−42.
38. Nicas, T. I., and Iglewski, B. H. (1984) *Infect. Immun. 45*, 470−4.
39. Woods, D. E., and Sokol, P. A. (1985) *Eur. J. Clin. Microbiol. 4*, 163−9.
40. Apodaca, G., Bomsel, M., Lindstedt, R., Engel, J., Frank, D., Mostov, K. E., and Wiener-Kronish, J. (1995) *Infect. Immun. 63*, 1541−51.
41. Kudoh, I., Wiener-Kronish, J. P., Hashimoto, S., Pittet, J. F., and Frank, D. (1994) *Am. J. Physiol. 267*, L551−6.
42. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis 18*, 2714−23.
43. Wang, B., Yang, H., Liu, Y., Jelinek, T., Zhang, L., Rouslahti, E., and Fu, H. (1999) *Biochemistry* (in press).

BI991019L