Raf-1 Kinase and Exoenzyme S Interact with 14-3-3ζ through a Common Site Involving Lysine 49*

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14-3-3 proteins are a family of conserved dimeric molecules that bind to a range of cellular proteins involved in signal transduction and oncogenesis. Our solution of the crystal structure of 14-3-3ζ revealed a conserved amphipathic groove that may allow the association of 14-3-3 with diverse ligands (Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) Nature 376, 191–194). Here, the contributions of three positively charged residues (Lys-49, Arg-56, and Arg-60) that lie in this Raf-binding groove were investigated. Two of the charge-reversal mutations greatly (K49E) or partially (R56E) decreased the interaction of 14-3-3ζ with Raf-1 kinase, whereas R60E showed only subtle effects on the binding. Interestingly, these mutations exhibited similar effects on the functional interaction of 14-3-3ζ with another target protein, exoenzyme S (ExoS), an ADP-ribosyltransferase from Pseudomonas aeruginosa. The EC50 values of 14-3-3ζ required for ExoS activation increased by ~110-, 5-, and 2-fold for the K49E, R56E, and R60E mutants, respectively. The drastic reduction of 14-3-3ζ/ligand affinity by the K49E mutation is due to a local electrostatic effect, rather than the result of a gross structural alteration, as evidenced by partial proteolysis and circular dichroism analysis. This work identifies the first point mutation (K49E) that dramatically disrupts 14-3-3ζ/ligand interactions. The parallel effects of this single point mutation on both Raf-1 binding and ExoS activation strongly suggest that diverse associated proteins share a common structural binding determinant on 14-3-3ζ.

14-3-3 proteins are a family of dimeric eukaryotic molecules involved in diverse cellular processes (see Ref. 1 for review). They were originally identified as brain-specific proteins (2) and later found to be present in most mammalian tissues as well as in other eukaryotic organisms, including yeast and plants. Members of the 14-3-3 family have been highly conserved throughout evolution (>60% identity between yeast and mammalian isoforms), reflecting the fundamental importance of 14-3-3 proteins in cellular physiology. Indeed, deletion of the two isoforms of yeast 14-3-3 is lethal to both Saccharomyces cerevisiae and Schizosaccharomyces pombe (3–5).

Multiple biochemical activities have been ascribed to the 14-3-3 family of proteins, although the precise function of 14-3-3 in cellular regulation is unclear. They have been shown to activate tyrosine and tryptophan hydroxylases (6, 7) and are involved in the regulation of protein kinase C activity (8–10). In vitro, 14-3-3 directly activates a bacterial ADP-ribosyltransferase, exoenzyme S (ExoS),1 from Pseudomonas aeruginosa (11). Because of its specificity and sensitivity, activation of ExoS has been used as a simple functional assay for the presence of 14-3-3 proteins (12). Recently, members of the 14-3-3 protein family were found to associate with a number of protooncogene and oncogene products (13) as well as with proteins involved in apoptosis. These 14-3-3-associated proteins include Raf kinases (12, 14–16), Bcr and Bcr-Abl (17), Cdc25 phosphatases (18), phosphatidylinositol 3-kinase (19), Cbl (20), the middle tumor antigen of polyoma virus (21), A20 (22), and Bad (23). Through protein/protein interactions, 14-3-3 may regulate diverse cellular processes.

The 14-3-3/Raf interaction has been extensively studied because of the central importance of Raf kinases in mitogenic signal transduction. Both in vivo and in vitro experimental data strongly support a biologically relevant interaction of 14-3-3 with Raf kinases. For instance, overexpression of either the yeast homologue, BMH1, or mammalian 14-3-3 stimulates the biological activity of mammalian Raf in budding yeast (14, 15). Additionally, microinjection of 14-3-3 mRNA into Xenopus oocytes promotes Raf-dependent oocyte maturation (16). Raf immunoprecipitates from yeast or oocytes in the above experiments have increased kinase activity. However, when Raf kinase activity is measured directly with purified 14-3-3 in vitro, no activation of Raf can be demonstrated (12, 24, 25). Consistent with this, Dent et al. (26) found that 14-3-3 proteins can block the dephosphorylation and inactivation of Raf kinase by phosphatases in vitro. Recent demonstration of oligomerization-induced Raf activation suggests an intriguing role of 14-3-3 in promoting Raf dimerization/oligomerization during the activation process (27, 28) because it is possible for one dimeric 14-3-3 molecule to bind two Raf kinase molecules (29, 30). A negative role of 14-3-3 in Raf regulation has also been postulated based on the observation that Raf-1 mutants unable to stably interact with 14-3-3 exhibit enhanced kinase activity in mammalian cells and Xenopus oocytes and are biologically active.

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1 The abbreviations used are: ExoS, exoenzyme S from P. aeruginosa; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; SBTI, soybean trypsin inhibitor; WT, wild-type.
activated (31). However, it remains elusive how 14-3-3 proteins contribute to Raf signaling pathways.

To understand the biological consequence of the 14-3-3/Raf interaction in vivo, it is important to determine the key residues of 14-3-3 required for Raf binding. Defined point mutants will provide essential tools for dissecting the role of 14-3-3 in Raf activation and in cellular regulation in general. Toward this goal, we have solved the three-dimensional crystal structure of the ζ-isoform of the 14-3-3 proteins (30). In the structure, each monomer of the dimeric protein consists of a bundle of nine α-helices organized in an antiparallel fashion. The four N-terminal α-helices participate in dimer formation. When viewed along the mean helix axis, the bundle of helices, α-helices 3-9, form a palisade. Residues that are conserved among members of the 14-3-3 family line the inside of this palisade and define a conserved groove. Not surprisingly, 14-3-3 proteins are also conserved at the tertiary structural level. The structure of 14-3-3z (29) exhibits a very similar folded conformation to the ζ-isoform, and this should facilitate the identification of common residues of 14-3-3 required for ligand binding. Inspection of the surface property of the conserved groove of the 14-3-3ζ monomer reveals an amphipathic structure that is lined with charged residues on one side and hydrophobic residues on the other (30). Based on the fact that 14-3-3 proteins bind to a wide range of cellular partners, it is possible that this conserved groove of 14-3-3ζ is involved in ligand binding.

To test the crystal structural model, we set about to systematically analyze the contribution of individual residues in the amphipathic groove to ligand binding. Here, we report the identification of the first point mutation (K49E) of 14-3-3 proteins that disrupts the association of 14-3-3ζ with Raf-1 kinase and diminishes its ability to activate ExoS. The impaired binding is not due to gross alteration of the mutant 14-3-3ζ protein based on our partial proteolysis and CD analysis. The concomitant effects of a single point mutation on both Raf binding and ExoS activation strongly suggest that diverse ligands may share a common site on 14-3-3 proteins and that Lys-49 may represent a general site for ligand binding.

MATERIALS AND METHODS
Cell Growth Conditions and DNA Manipulations
Escherichia coli strains were grown routinely at 37 °C in LB broth or on LB agar plates (15 g/l). Ampicillin was used as required. Plasmids were routinely grown at 37 °C in LB broth or on LB agar plates (15 g/l). Ampicillin was used as required. Exonuclease III was used for partial restriction enzyme digestion. The following primers were used as described (33).

Raf sequences were amplified by polymerase chain reaction with added restriction sites using a full-length raf gene as a template (33). The following primers were used: 1) 5’-GGATCCGAATTCCTAGAAGGAGAATCTCAATGGAATAAAAAAGAAGAGCTG-3’ (EcoRI and Ndel sites underlined) and 5’-CTCATGAAATTCCTGACTCATTATTTCCTCCCT-3’ (EcoRI and SalI sites underlined). The amplified DNA fragments were digested with EcoRI and subcloned into pUC19 at the EcoRI site with the 14-3-3ζ gene in the opposite orientation of lacZ. To introduce appropriate restriction sites for in-frame fusion of various versions of Raf-1 kinase, we constructed three basic Raf vectors. For this purpose, Raf sequences were amplified by polymerase chain reaction with added restriction sites using a full-length raf gene as a template (33).

Vectors for Yeast Two-hybrid/Interaction Trap System—Parental vectors for the interaction trap system were kind gifts from Dr. Roger Brent (Harvard Medical School) and included pEG202 and pJG4-5 (34). pEG202 contains the sequence encoding the bacterial LexA protein, the expression of which is under the control of the ADH promoter. pJG4-5 carries the B42 transcriptional activation sequence fused to a nuclear localization sequence under control of the GAL1 promoter. An epitope tag (influenza virus hemagglutinin) was inserted between the nuclear localization sequence and the sequence of interest to enable surveillance of the expressed fusion protein by immunoblotting.

Expression Vectors—For protein expression in E. coli, the Ndel/EcoRI fragments of pHAF625 encoding the mutant 14-3-3ζ proteins (see below) were subcloned into Ndel-EcoRI-cut pET-15b (Novagen, Madison, WI), generating plLZ121 (K49E), phW167 (K49L), phW168 (K49Q), phW169 (K49R), plLZ125 (R56E), and plLZ126 (R60E).

Site-directed Mutagenesis
Oligonucleotide-directed mutagenesis was performed with double-stranded plasmid as the starting material according to the unique site elimination method (35). Two primers were used in this process, a mutagenic primer containing a desired mutation and a selection primer containing a mutation that eliminated a unique SspI site (36). The resulting plasmid was cotransformed with pEG202 derivatives and pJG4-5 derivatives by the lithium acetate method (36).

Yeast Two-hybrid/Interaction Trap Assay
A modified version of the yeast two-hybrid system (36), the interaction trap (34), was used for studying 14-3-3/Raf interactions in vivo (33). E. coli strains EGY48 (MATa trp1 ura3 his3 LEU2::LexAop6-LEU2) was used as a host for all interaction experiments. Both pHS18-34 and pJK103 were used as reporters. pHS18-34 directs expression of a GAL1-lacZ gene from high affinity ColdLexA operators, whereas pJK103 uses two ColdLexA operators. For the protein/protein interaction analysis, EGY48 harboring a lacZ reporter was cotransformed with pEG202 derivatives and pJG4-5 derivatives by the lithium acetate method (33). Transformants were maintained in synthetic medium with glucose (2%) under selection for the URA3, HIS3, and TRP1 markers. Colonies were patched onto synthetic medium plates containing galactose (2%), raffinose (1%), and X-gal (Gold...
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Biotechnology, Inc., St. Louis, MO) to induce and detect the expression of the lacZ reporter gene. The time required for color development of positive interactions on X-gal selection plates ranged from 8 to 24 h.

Assay of β-Galactosidase Activity

For the quantitative liquid assay, yeast transformants were grown overnight in the appropriate synthetic selective medium with galactose to $A_{600} = 1.0$. Cells were washed with and transferred to the galactose induction medium with 1% raffinose. After 12 h of induction, cells were prepared in fresh Z buffer and permeabilized using three drops of chloroform and two drops of 0.1% SDS as described (33). For quantitation, chlorophenol-red-β-D-galactopyranoside (Boehringer Mannheim) was added as a chromogenic substrate. The amount of liberated chlorophenol red was determined by $A_{492}$, and the specific activity in Miller units was calculated based on the following equation: unit = $(A_{492}/A_{6000} \times V \times T) / 1000$, where $A_{6000}$ is the cell density used, $V$ is the volume of the culture in ml, and $T$ is the reaction time in min. Three independent colonies were used for each activity determination.

Protein Expression and Purification

Single colonies from LB/ampicillin plates, freshly streaked with E. coli BL21(DE3) harboring pET-15b-derived plasmids expressing 14-3-3ζ or its mutant derivatives, were transferred into 3-ml cultures of LB/ampicillin. These cultures were fermented overnight at 37 °C on a rotary wheel. On the second day, 2-liter flasks, each containing 250 ml of LB/ampicillin, were inoculated with the 3-ml overnight culture. The flasks were aerated on a rotary shaker (300 rpm, Innova 4000, New Brunswick Scientific, Edison, NJ) at 37 °C. The cultures, containing 9% sucrose, 0.5% sodium deoxycholate, 0.1% SDS, 137 mM NaCl, and 20 mM Tris-HCl (pH 8.0), were sonicated four times on ice for 20 s followed by centrifugation at 4000 $g$ for 10 min at 4 °C. The pellets were washed twice with ice-cold sodium acetate buffer composed of 50 mM Na$_2$HPO$_4$ (pH 8.0), 100 mM KCl, 0.1% Tween 20, 1.0 mM phenylmethylsulfonyl fluoride, and 1.0 mM EDTA. The resuspended pellets were sonicated four times on ice for 20 s each using a Branson 450 Sonifier cell disruptor, with the power control maintained along the groove, exposed residues Lys-49, Arg-56, and Arg-60 are charged residues project toward the inside of the groove, which

The far-UV CD spectra were recorded on a Jasco 574 spectropolarimeter. Results are expressed as mean residue molar ellipticity $(\theta)$, degree cm$^2$dg$^{-1}$, calculated from the following equation: $\theta = -10,000 \times MRW/\Delta L C$, where $\Delta L$ is the observed ellipticity expressed in millidegree, MRW is the mean residue molecular weight (114 for 14-3-3ζ), $C$ is the final protein concentration in mg/ml. Far-UV CD spectra were the average of four scans obtained by collecting data at 0.2-nm intervals from 250 to 190 nm with a protein concentration of 0.4 mg/ml in phosphate-buffered saline. For the guanidine hydrochloride denaturation experiments, changes in the helical content of the sample were monitored at 222 nm. Phosphate-buffered saline was used for these studies. The half-melting concentration ($C_{1/2}$) is defined as the concentration of denaturant at the midpoint of the transition. The breadth of the transition ($AC$) is defined as the difference in denaturant concentrations at the points at which the transition is one-fourth and three-fourths complete.

SDS-PAGE and Immunoblotting

An SDS-PAGE system was used essentially as described by Laemmli (38). The enzyme-linked immunoblotting procedures of Towbin et al. (39) were followed. Proteins separated by SDS-PAGE were electrothermically transferred to nitrocellulose membranes and incubated with antiserum (1:2000 dilutions for mouse anti-hemagglutinin monoclonal antibody (BACCO, Richmond, CA)). Cross-reacting materials were visualized with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G using ECL (Amersham Corp.).

Protein Assay

Protein concentration was estimated by the Bio-Rad protein assay kit based on the method of Bradford (40), and bovine serum albumin was used as the standard.

RESULTS

Crystal Structure of 14-3-3ζ Suggests the Participation of Charged Residues in Ligand Binding—In the three-dimensional crystal structure of 14-3-3ζ, we identified a conserved amphipathic groove that may represent a general site for binding to associated proteins (Fig. 1) (30). On the charged face of the groove, exposed residues Lys-49, Arg-56, and Arg-60 are lined along α-helix 3. The side chains of these three positively charged residues project toward the inside of the groove, which

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positions the charged groups of the side chains well for potential contact with charged groups from the interacting ligand. As a first step in the systematic dissection of residues of 14-3-3 involved in ligand binding, we examined the contributions of these three positively charged residues to 14-3-3 protein/protein associations.

Mutations of Lys-49, Arg-56, and Arg-60 Decrease the Binding of 14-3-3ζ to Raf-1 Kinase—To test whether charged residues in the proposed groove are involved in ligand binding, we made three charge-reversal mutations by site-directed mutagenesis, generating K49E, R56E, and R60E (Fig. 1). It has been established that 14-3-3ζ directly binds to Raf-1 kinase in vivo and in vitro (13). Individual mutations were tested for their effect on the interaction of 14-3-3ζ with Raf-1 kinase.

We used the interaction trap to determine the 14-3-3ζ/Raf interaction in an in vivo environment (34). Raf-1 was fused to the DNA-binding domain of LexA protein, whereas 14-3-3ζ was fused to the B42 transcriptional activation sequence as described under “Materials and Methods.” Interaction between the LexA-Raf-1 and B42–14-3-3ζ fusion proteins results in transcription of a lacZ reporter and consequently β-galactosidase activity that can be visualized using chromogenic substrates (X-gal). Initially, the X-gal plate assay was performed to assess the relative interactions of 14-3-3ζ and Raf in various combinations. Consistent with previous reports (14–16), expression of 14-3-3ζ and Raf-1 induced the production of β-galactosidase activity (Table I), reflecting the interaction of 14-3-3ζ with Raf-1 in yeast. No β-galactosidase activity was detectable when LexA-Raf-1 or B42–14-3-3ζ fusion protein “Materials and Methods.” Interaction between the LexA-Raf-1 and B42–14-3-3ζ fusion proteins results in transcription of a lacZ reporter and consequently β-galactosidase activity that can be visualized using chromogenic substrates (X-gal). Initially, the X-gal plate assay was performed to assess the relative interactions of 14-3-3ζ and Raf in various combinations. Consistent with previous reports (14–16), expression of 14-3-3ζ and Raf-1 induced the production of β-galactosidase activity (Table I), reflecting the interaction of 14-3-3ζ with Raf-1 in yeast. No β-galactosidase activity was detectable when LexA-Raf-1 or B42–14-3-3ζ fusion protein was expressed independently. Introduction of the charge-reversal mutations K49E and R56E abolished β-galactosidase production, suggesting decreased binding of 14-3-3ζ to Raf-1. The mutation R60E had no significant effect on the 14-3-3ζ/Raf interaction in this assay. It has been demonstrated that 14-3-3 binds to both the N-terminal regulatory fragment of Raf-1 and the C-terminal kinase domain (12, 14–16, 25). Consistent with these reports, cotransfection of yeast with WT 14-3-3ζ and either the N- or C-terminal sequence of Raf-1 induced blue colony formation (Table I). Interestingly, the K49E and R56E mutations diminished the interaction of 14-3-3ζ with both the N- and C-terminal domains. This result suggests that the N- and C-terminal fragments of Raf-1 associate with 14-3-3ζ via a similar site on 14-3-3ζ and that 14-3-3ζ can interact with the N- or C-terminal fragment of Raf-1 independently. To rule out possible reporter effects (41), two types of lacZ reporters were used for the above experiments: a sensitive reporter, pSH18-34 (with eight operators for LexA binding), and a less sensitive reporter, pJK103 (with two operators). Similar results were obtained with each (data not shown).

To quantify the effect of mutations on 14-3-3ζ/Raf interactions, the above experiments were further performed using a quantitative liquid assay. As shown in Fig. 2, 14-3-3ζ bound to the full-length or truncated forms of Raf-1 with different affinities, with Raf-N being strongest and Raf-C weakest (16). Consistent with the above assay, the mutation K49E completely abolished the interaction of 14-3-3ζ with both the N- and C-terminal domains. This result suggests that the N- and C-terminal fragments of Raf-1 associate with 14-3-3ζ via a similar site on 14-3-3ζ and that 14-3-3ζ can interact with the N- or C-terminal fragment of Raf-1 independently. To rule out possible reporter effects (41), two types of lacZ reporters were used for the above experiments: a sensitive reporter, pSH18-34 (with eight operators for LexA binding), and a less sensitive reporter, pJK103 (with two operators). Similar results were obtained with each (data not shown).

To confirm the results obtained with the interaction trap system, an alternative in vitro binding assay was performed. For this purpose, WT and mutant 14-3-3ζ proteins were overex-

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**Table I**

| LexA fusion protein | B42 fusion protein |
|---------------------|-------------------|
| 14–3-3ζ             | Raf-1             | Raf-N | Raf-C | B42 alone |
| LexA alone           | –                 | –     | –     | –         |
| WT 14–3-3ζ           | +                 | +     | +     | +         |
| Mutant 14–3-3ζ       | +                 | +     | +     | +         |
| K49E                 | +                 | –     | –     | –         |
| R56E                 | +                 | +     | +     | +         |
| R60E                 | +                 | +     | +     | +         |
| K49L                 | +                 | +     | +     | +         |
| K49Q                 | +                 | +     | +     | +         |
| K49R                 | +                 | +     | +     | +         |

*The blue color was very weak just above the background.*
pressed in *E. coli* as hexahistidine-tagged fusion proteins and immobilized on nickel-charged beads. The binding partner, Raf-1, was expressed and labeled with [35S]methionine using the TNT *in vitro* transcription-translation system. Equal amounts of radiolabeled Raf-1 protein mixtures were incubated individually with the WT or mutant 14-3-3ζ-coated beads. After extensive washing, Raf-1 protein that was complexed with various 14-3-3 derivatives was visualized following SDS-PAGE separation and autoradiography (Fig. 3A). The lower panels show the relative levels of the WT and mutant 14-3-3ζ proteins expressed in test strains, and for these experiments, equivalent amounts of total cell protein were analyzed. Anti-hemagglutinin monoclonal antibody (HA.11) was used to probe the B42–14-3-3ζ sequences in the pJG4-5 vectors. Control strains contain pEG202 (carrying the respective *raf* genes) in combination with the pJG4-5 plasmid. The expression levels of Raf proteins are similar in all samples.

**Mutations of Lys-49, Arg-56, and Arg-60 Decrease the Ability of 14-3-3ζ to Activate ExoS—**Data from the above experiments suggested the participation of Lys-49 and Arg-56, and possibly Arg-60, in the 14-3-3ζ/Raf interaction. However, it is unknown whether Lys-49 and Arg-56 are specific to the Raf interaction or whether they form part of a general interaction surface for multiple ligands. To address this question, we used a defined biochemical assay, the ExoS activation reaction (11). ExoS is an ADP-riboseyltransferase that catalyzes, in a 14-3-3-dependent manner, the incorporation of the ADP-ribose moiety of the NAD+ molecule into substrates, including Ras, vimentin, and SBTI (11, 42).

For the kinetic studies and protein stability analysis, 14-3-3ζ and its mutants were overexpressed in *E. coli* BL21(DE3), and individual proteins were purified by nickel chelate affinity chromatography. Hexahistidine tags were removed by enzymatic digestion. The proteins were further purified by gel filtration chromatography on a Superdex 200 column and reached ~95% purity (data not shown).

The recombinant WT 14-3-3ζ protein activated ExoS to ADP-ribosylate SBTI in a dose-dependent manner, with an EC<sub>50</sub> of 3.0 nM (Table II). The specific incorporation of ADP-ribose into the substrate SBTI was confirmed by an SDS-PAGE-based assay (see “Materials and Methods”; data not shown). As in the case of Raf binding, K49E drastically increased the EC<sub>50</sub> of 14-3-3ζ in the ExoS assay (334 nM, ~110-fold increase), suggesting the decreased affinity of this 14-3-3 mutant toward ExoS. The R56E mutation partially decreased the ability of 14-3-3ζ to activate ExoS, as measured by the amount of 14-3-3 needed to achieve 50% of *V*<sub>max</sub> (EC<sub>50</sub> ~ 15.9 nM) (Table II). Again, R60E had only a slight effect on ExoS activation. None of these mutants, however, affected the *V*<sub>max</sub> of the reactions (Table II). Therefore, there is a direct correlation between the effects of mutations of Lys-49, Arg-56, and Arg-60 on interaction with Raf-1 and activation of ExoS.

**Panel of Lys-49 Mutations Suggests a Role for the Positive Charge in Ligand Binding—**Among three mutants tested above, K49E exhibited the most severe defect in 14-3-3ζ/ligand

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**Fig. 2. Effect of mutations of Lys-49, Arg-56, and Arg-60 of 14-3-3ζ on its interaction with full-length Raf-1 kinase, Raf-N, and Raf-C in yeast.** Strains EGY48 harboring pSH18-34, the pEG202 expression vector (carrying LexA-Raf) and the pJG4-5 plasmid (carrying B42–14-3-3ζ or its mutant derivatives) were grown in synthetic medium with galactose. Cells (1 × 10<sup>7</sup>) were collected and divided for the β-galactosidase assay described as above in “Materials and Methods” (upper panels) and for immunoblotting analysis (lower panels). The vertical bars represent relative β-galactosidase activities expressed (n = 4). The lower panels show the relative levels of the WT and mutant 14-3-3ζ proteins expressed in test strains, and for these experiments, equivalent amounts of total cell protein were analyzed. Anti-hemagglutinin monoclonal antibody (HA.11) was used to probe the B42–14-3-3ζ proteins because an influenza virus hemagglutinin epitope (HA) was inserted between the B42 and 14-3-3ζ sequences in the pJG4-5 vectors. Control strains contain pEG202 (carrying the respective *raf* genes) in combination with the pJG4-5 plasmid. The expression levels of Raf proteins are similar in all samples.

**Fig. 3. Comparison of *in vitro* binding of Raf-1 kinase to WT and mutant 14-3-3ζ proteins.** Transcripts of Raf-1 kinase were generated from the T7 promoter in pHW102. Raf-1 proteins were synthesized *in vitro* in a rabbit reticulocyte lysate in the presence of [35S]methionine. Equal portions of the labeled Raf protein were incubated with hexahistidine-tagged 14-3-3ζ or mutant derivatives bound to Sepharose beads for 1 h at 4°C. The immobilized WT or mutant 14-3-3ζ complexes were washed extensively (see “Materials and Methods”), and the bound proteins were eluted with sample buffer, analyzed by SDS-PAGE (12.5%), and visualized using a PhosphorImager. Hexahistidine-tagged β-galactosidase protein bound to beads was used as a control. 14-3-3ζ/ExoS interactions were tested using the mutant 14-3-3ζ proteins K49E, R56E, and R60E (A) or a panel of Lys-49 mutants (B). The amounts of immobilized proteins (β-galactosidase (β-Gal) and 14-3-3ζ) used were revealed by Coomassie Blue staining.

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binding. The nature of the contribution of Lys-49 to ligand binding was further probed by changing Lys-49 to Leu, Gln, or Arg. This panel of Lys-49 mutants was overexpressed in E. coli and purified as described under "Materials and Methods." The effect of these Lys-49 mutations on 14-3-3-\(\zeta\)/ligand binding was investigated using the Raf binding and ExoS activation assays described above. In comparison with K49E, a neutral Leu substitution slightly increased the binding affinity of 14-3-3-\(\zeta\) for Raf as measured in the interaction trap system (Fig. 4) and the in vivo binding assay (Fig. 3B). A polar Gln residue and a positively charged Arg residue significantly restored the binding activity (Fig. 4). The ability of this panel of 14-3-3 mutants to activate ExoS was also examined and was found to increase in the following order in relation to the K49E mutant: K49L to activate ExoS was also examined and was found to increase in the following order in relation to the K49E mutant: K49L < K49Q < K49R (Table II). It appears that a positive charge is important for both Raf binding and ExoS activation. It is interesting that the affinities of the Lys-49 mutants for Raf-1 kinase directly paralleled the efficiencies of ExoS activation, supporting the notion that Raf-1 and ExoS bind to 14-3-3-\(\zeta\) through a common site.

**K49E, R56E, and R60E Mutations Do Not Result in Gross Structural Changes in 14-3-3-\(\zeta\)—**To explore the possibility that the reduced activity of mutant 14-3-3 proteins is a result of structural changes, we probed their structural integrity using several alternative approaches. First, the overall structural integrity of the 14-3-3 mutants was examined by assessing their ability to dimerize. Crystal structure data (29, 30) and other biochemical analyses (43, 44) suggest that 14-3-3 proteins function as a dimer. We found that dimer formation can be demonstrated in vivo using the interaction trap system (Table II). This observation provided a simple means to evaluate the relative conformational integrity of the mutant proteins in vivo because dimer formation depends on the integrity of the folded proteins (45). Despite specific defects in association with Raf-1 and activation of ExoS, each of the 14-3-3 mutants readily formed dimers with the WT (Table I) and mutant (data not shown) partners in vivo. In vitro, the purified 14-3-3 mutant proteins comigrated with the WT protein, as a dimer, on a native gel and on a gel filtration column (Superdex 75 HR10/30) (data not shown). These data suggest that the Lys-49 mutations, R56E, and R60E caused no drastic perturbation to the overall structure of the proteins.

Second, the relative stability of the mutant proteins compared with that of WT 14-3-3-\(\zeta\) was evaluated by analysis of the proteins’ resistance to limited proteolytic digestion with chymotrypsin. Purified WT 14-3-3-\(\zeta\) and its mutant derivatives were incubated with chymotrypsin for 30 min, followed by analysis of the digestion products by SDS-PAGE and silver staining. The mutant proteins gave similar digestion patterns compared with WT 14-3-3-\(\zeta\) (Fig. 5), suggesting that the global structure of each mutant protein was not altered. Similar results were obtained using additional proteases, including Protease and carboxypeptidase B.

Third, as a quantitative measure of the possible structural effect of mutations, we used CD spectroscopy to compare the overall secondary structure of the mutant 14-3-3 proteins with that of the WT protein. Consistent with the three-dimensional crystal structure (29, 30) and earlier reports (46), the far-UV CD spectra of 14-3-3-\(\zeta\) proteins were characteristic of an \(-\alpha\)-helical structure (Fig. 6). Ellipticity minima were observed near 208 and 222 nm. As shown in Fig. 6, the Lys-49 mutations, R56E, and R60E resulted in virtually no effect on the CD spectrum of 14-3-3-\(\zeta\), suggesting that these mutations do not alter the global secondary structure of 14-3-3-\(\zeta\). Because K49E causes the most dramatic reduction in 14-3-3-\(\zeta\)/ligand binding, we further compared the chemical stability of the K49E mutant and the WT protein as measured by CD at 222 nm. Strikingly, the K49E mutant, which disrupted the 14-3-3-\(\zeta\)/Raf interaction and drastically decreased the affinity for ExoS, was as stable as the WT protein as judged by the guanidine hydrochloride-induced unfolding profiles. The midpoint denaturation concentrations and the concentration intervals in which chemical denaturation took place did not change for K49E proteins (WT: \(C_m = 1.31 \pm 0.10 \text{ M}\), \(\Delta C = 0.25 \pm 0.03 \text{ M}\); K49E: \(C_m = 1.35 \pm 0.13 \text{ M}\), \(\Delta C = 0.21 \pm 0.05 \text{ M}\)). Similarly, no change in the \(C_m\) and \(\Delta C\) values of other Lys-49 mutants was observed. Taken to-

**TABLE II**

*Summary of kinetic parameters for ExoS activation by WT and mutant 14-3-3-\(\zeta\) proteins*

| 14-3-3-\(\zeta\) | \(V_{max}\) pmol ADP-ribose incorporated | EC\(50\) µM | Relative EC\(50\) |
|----------------|--------------------------------------|-----------------|-----------------|
| WT             | 5.54 ± 0.64                          | 3.0 ± 0.2       | 1.0             |
| K49E           | 5.49 ± 0.70                          | 334 ± 35        | 113.3           |
| K49L           | 5.47 ± 0.54                          | 261.1 ± 2.4     | 8.7             |
| K49Q           | 5.47 ± 0.59                          | 10.4 ± 0.9      | 3.5             |
| K49R           | 5.49 ± 0.58                          | 3.5 ± 0.3       | 1.2             |
| R56E           | 5.47 ± 0.56                          | 15.9 ± 1.4      | 5.3             |
| R60E           | 5.49 ± 0.61                          | 6.0 ± 0.6       | 2.0             |

2. S. Masters, L. Zhang, and H. Fu, unpublished results.

![Figure 4](http://www.jbc.org/ by guest on July 25, 2018)
14-3-3 proteins interact with diverse proteins involved in a wide range of biological systems (1). Based on our crystal structure data, we proposed that 14-3-3 binds to multiple ligands through a conserved amphipathic groove (30). On the charged face of the groove, three positively charged residues (Lys-49, Arg-56, and Arg-60) are lined along α-helix 3. Here, we report that charge-reversal mutations at these three positions disrupted the interaction of 14-3-3 with Raf-1 kinase and ExoS to different degrees, with K49E being the most dramatic. The disruption of the 14-3-3/ligand interaction by mutations of Lys-49 appears to be due to the local alteration of the charge property at this position, rather than mutation-induced structural change. More important, our work identifies a critical residue, Lys-49, with general importance for ligand binding, which is consistent with the crystal structure model. In addition, application of a panel of Lys-49 mutations establishes a clear correlation between the disruption of 14-3-3/Raf association and the 14-3-3/ExoS interaction, suggesting that diverse associated proteins use a common binding site on 14-3-3. In further support of this notion, the effective association of 14-3-3 with the N-terminal fragment of c-Bcr kinase (encoded by the first exon) was also disrupted by the K49E mutation (data not shown).

Lys-49 appears to be part of a binding interface in which multiple residues are involved. Clearly, Arg-56 also contributes to ligand binding because the charge-reversal mutation of Arg-56 partially decreased the binding of 14-3-3 to Raf and ExoS. Only a slight effect of the R60E mutation on ligand binding was observed. The mechanism by which Lys-49 contributes to ligand binding was investigated by replacing the initial charge-reversal mutation residue (Glu) with Leu, Gln, or Arg. It appears that the hydrophobic nature of the Lys-49 side chain is not the primary determinant for 14-3-3 binding since a neutral Arg participates in ligand binding. It is also conceivable that an initial contact at the Lys-49 region may induce a conformational change that is required to expose the primary binding site in the C-terminal fragment of 14-3-3.

The data presented here support the notion that 14-3-3 uses the conserved amphipathic groove for binding to multiple ligands, which include Raf, Ber, and ExoS. A complementary amphipathic conformation in the ligand may permit the specific interaction of 14-3-3 with diverse associated proteins. For instance, the phosphorylation in ligands may provide part of the charged interface for 14-3-3 binding since a hydrophobic Leu residue showed only a slight effect. The ability of 14-3-3 to bind Raf was partially restored with the elimination of the negative charge and the introduction of Gln or Arg, suggesting that the positive charge of Lys-49 is involved in the association of 14-3-3 with Raf kinase. However, at this point, we cannot rule out the possibility that Lys-49 is indirectly involved in ligand binding, for example, by interacting with residues in the C-terminal loop of the 14-3-3 protein. In our crystal structure, the C-terminal 12 amino acid residues form a poorly ordered structure and appear lined in the proposed ligand-binding groove (30).

Drastic deletion analysis of 14-3-3 implicates a different region of the protein in ligand binding. Luo et al. (47) reported the importance of the C-terminal sequence (amino acids 179–245) of 14-3-3 in the binding of Raf-1 kinase in COS cells. Interestingly, only the inactive form of Raf kinase was found associated with this C-terminal 14-3-3 fragment, and the active Raf form was complexed only with the full-length 14-3-3 protein. A C-terminal fragment of 14-3-3 (residues 166–208; termed BoxI) was also found to be necessary and sufficient for association with phosphorylated tyrosine hydroxylase (48). However, Liu et al. (20) found that deletion of the N-terminal sequence, which abolished dimer formation, also impaired the binding of 14-3-3 to Raf, Cbl, and p85 (phosphatidylinositol 3-kinase). It is possible that the N-terminal sequence directly or indirectly participates in ligand binding. It is also conceivable that an initial contact at the Lys-49 region may induce a conformational change that is required to expose the primary binding site in the C-terminal fragment of 14-3-3.

As a complementary study, we performed a structural analysis of 14-3-3 proteins. CD spectra were generated at 25 °C using 0.4 mg/ml protein in phosphate-buffered saline.

FIG. 5. Comparison of partial protease digestion patterns of WT and mutant 14-3-3 proteins. 2.5 μg of purified 14-3-3 proteins were incubated with 0.5 unit of chymotrypsin for 30 min at 37 °C. Digested proteins were separated on SDS-PAGE (15%) and revealed by silver staining.

FIG. 6. Far-UV CD spectra of WT and mutant 14-3-3 proteins. CD spectra were generated at 25 °C using 0.4 mg/ml protein in phosphate-buffered saline.
sibility that 14-3-3, like calmodulin, may regulate the activity of the associated proteins directly. Evidence is accumulating. The first example is the stimulatory role of 14-3-3 for tyrosine and tryptophan hydroxylases after phosphorylation by Ca$^{2+}$/calmodulin-dependent kinase II (6). 14-3-3 has also been found to inhibit (8) or activate (9) protein kinase C and serves as an obligatory activator of ExoS ADP-ribosyltransferase (11). Interestingly, 14-3-3 proteins serve as inhibitors of nitrate reductase from spinach leaves in a phosphorylation-dependent manner (52). However, a direct role of 14-3-3 in Raf kinase activation seems unlikely because the addition of purified 14-3-3 to the Raf protein has no significant effect (12, 24, 25, 31).

14-3-3 proteins have been shown to interact with Raf kinases and are implicated in Raf-mediated signal transduction. Earlier experiments have demonstrated that 14-3-3 binds to Raf via multiple sites. In support of this notion, two phosphorylation-dependent high affinity binding sites in Raf have been identified, which are the N-terminal regulatory domain sequence containing Ser-259 and the C-terminal kinase domain sequence containing Ser-621 (49). Consistent with these data, we show here that 14-3-3 can bind independently to the Raf-N and Raf-C fragments (Fig. 2). Moreover, the point mutation K49E abolished the binding of 14-3-3 to either Raf-N or Raf-C. We conclude that Raf-N and Raf-C utilize a similar structural determinant on 14-3-3 for binding that involves Lys-49. Such an interaction could enable a single 14-3-3 dimer to bind to the N- and C-terminal domains of the same Raf molecule simultaneously to or bind to two molecules of Raf. Alternatively, it also allows one 14-3-3 dimer to tether one molecule of Raf and another 14-3-3-associated partner together.

Because Lys-49 is conserved among all 14-3-3 isoforms, Lys-49 equivalent residues in other isoforms may also participate in ligand binding in a similar fashion. However, isoform specificity and ligand specificity issues have not been addressed by current studies. It is possible that residues that are not conserved in 14-3-3 and/or binding sequences in the target proteins confer different levels of binding specificity.

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