Mutations in the Hydrophobic Surface of an Amphipathic Groove of 14-3-3ζ Disrupt Its Interaction with Raf-1 Kinase*

(Received for publication, November 26, 1997, and in revised form, March 4, 1998)

Haining Wang‡§, Lixin Zhang‡, Robert Liddington¶, and Haian Fu‡

From the ‡Department of Pharmacology and the §Graduate Program of Cell and Developmental Biology, Emory University School of Medicine, Atlanta, Georgia 30322 and the ¶Department of Biochemistry, University of Leicester, Leicester LE1 7RH, United Kingdom

14-3-3 proteins bind to a diverse group of regulatory molecules such as Raf-1, Cbl, and c-Bcr that are involved in signal transduction pathways. The crystal structure of 14-3-3ζ reveals a conserved amphipathic groove that may mediate the association of 14-3-3 with diverse ligands. Consistently, mutations on the charged surface of the groove (Lys-49, Arg-56, and Arg-60) decrease the binding of 14-3-3ζ to the ligands tested (Zhang, L., Wang, H., Liu, D., Liddington, R., and Fu, H. (1997) J. Biol. Chem. 272, 13717–13724). Here we report that mutations which altered the hydrophobic property of the groove, V176D, L216D, L220D, and L227D, disrupted the interaction of 14-3-3ζ with Raf-1 kinase. The reduced binding of the 14-3-3ζ mutants to Raf-1 was apparently not because of gross structural changes in the mutants as judged by their ability to form dimers, by partial proteolysis profiles, and by circular dichroism analysis. These hydrophobic residues appeared to be required for the binding of 14-3-3ζ to distinct activation states of Raf-1 because mutations V176D, L216D, L220D, and L227D reduced the interaction of 14-3-3ζ with Raf-1 from both phorbol 12-myristate 13-acetate-stimulated and unstimulated Jurkat T cells. These same mutations also disrupted the association of 14-3-3ζ with other regulatory molecules such as Cbl and c-Bcr, suggesting that the hydrophobic surface of the amphipathic groove represents part of a binding site shared by a number of 14-3-3-associated proteins. The conservation of the hydrophobic residues Val-176, Leu-216, Leu-220, and Leu-227 among known 14-3-3 family members implies their general importance in ligand binding.

A striking feature of 14-3-3 proteins is their ability to bind a wide range of cell regulatory proteins. Many of the 14-3-3-associated proteins are proto-oncogene or oncogene products that are known to participate in various signal transduction pathways leading to cell proliferation, differentiation, or transformation (Ref. 2 and references therein). These proteins include Raf kinases (4–7), c-Bcr and Bcr-Abl (8), Cbl (9), phosphatidylinositol 3-kinase (10), protein kinase C (11), Cdc25 phosphatases (12, 13), and the middle tumor antigen of polyoma virus (14). Proteins that interact with 14-3-3 are not restricted to proto-oncogene and oncogene products because proteins such as the glucocorticoid receptor (15), insulin-like growth factor I receptor (16), glycoprotein Ib (17), and epithelial keratins (18) are found to associate with 14-3-3. Two proteins that are involved in apoptosis, Bad (19) and A20 (20), have also been shown to interact with 14-3-3. Through its interaction with target proteins, 14-3-3 may participate in multiple cellular processes regulating cell proliferation, differentiation, and apoptosis. 14-3-3-target protein interactions appear to be regulated. The binding of 14-3-3 to Raf-1, c-Bcr, Cbl, and a number of other proteins is phosphorylation-dependent (9, 21, 22). Muslin et al. (23) identified a phosphoserine-containing consensus motif, RSXPXP (pS is a phosphorylated Ser), that serves as a 14-3-3 recognition site on several proteins. Like the interaction of phosphotyrosine motifs with SH2 domain-containing proteins, such a phosphoserine motif may provide a general mechanism for 14-3-3 protein-target interactions. In support of this notion, mutations of Raf-1 at Ser-259 of the RSXPXP motif disrupt the Raf-1-4-3-3 interaction (21). Double mutations at Ser-112 and Ser-136 in the 14-3-3-binding motifs of Bad, a proapoptotic protein, are shown to abolish the Bad-14-3-3 interaction and accelerate cell death (19). Other novel or variant motifs that recognize 14-3-3 have also been identified or implicated and include the Ser-rich motif of Cbl (24), the Ser-rich region of glycoprotein Ib (17), the Cys-rich domain of Raf-1 (Raf-CRD, residues 139–184) (25), and an unphosphorylated site in the bacterial ADP-ribosyltransferase, ExoS (26). Whether the 14-3-3-binding site of these proteins shares any structural similarity with the RSXPXP motif awaits further investigation.

The interaction of 14-3-3 with Raf-1 kinase has attracted much interest because of the central importance of Raf-1 kinase in mitogenic signal transduction and development (27). Two consensus 14-3-3-binding motifs have been identified in...
Raf-1 (23), one of which is localized in the N-terminal regulatory domain with Ser-259 as the phosphorylation site and the other of which located in the C-terminal kinase domain involving Ser-621. Recently, Raf-CRD, the Cys-rich domain within the regulatory fragment of Raf-1, has also been found to bind directly to 14-3-3ζ (25). However, how 14-3-3 binding contributes to Raf-1 activation remains unclear. It has been postulated that 14-3-3 may have dual functions (27). 14-3-3ζ may function to stabilize the inactive Raf-1 conformation possibly through its binding to the phosphorylated Ser-259 and Raf-CRD sites. Consistently, mutations in Ser-259 and Raf-CRD that impair Raf-1–14-3-3 interaction enhance Raf-1 activity (21, 25). During Raf-1 activation, 14-3-3ζ may also stabilize an active conformation of Raf-1 through its binding to the phosphorylated Ser-621 site. Other postulated mechanisms of 14-3-3-Raf-1 interactions include 14-3-3ζ-induced oligomerization of Raf-1 (28) and protection of Raf-1 from phosphatase-mediated inactivation (29). It is possible that the regulated interaction of 14-3-3ζ with distinct recognition sites on Raf-1 modulates the activation status of the Raf-1 kinase.

To understand how 14-3-3 proteins interact with diverse ligands and ultimately to determine the biological function of 14-3-3 protein-target interactions, we have solved the three-dimensional crystal structure of the ζ-isoform of 14-3-3ζ (30) and carried out mutational analysis to identify structural elements that are required for ligand binding (31). The 14-3-3ζ structure is solved as a dimer with a bundle of nine antiparallel α-helices in each monomer. When viewed along the mean helix axis, α-helices 3–9 form a palisade generating a groove. Interestingly, residues lining the inside of the groove are conserved among members of the 14-3-3 protein family. Within this conserved groove of the 14-3-3ζ monomer are several charged residues on one side and hydrophobic residues on the other (30) forming an amphipathic structure. This amphipathic property appears conserved in the ζ-isoform of the 14-3-3ζ protein as revealed by its crystal structure (32). It is possible that this conserved amphipathic groove allows the binding of 14-3-3ζ to diverse ligands. Consistent with this model, charge reversal mutations on one side of the groove, K49E, R56E, and R60E, greatly or partially decrease the interaction of 14-3-3ζ with Raf-1 kinase (31). Interestingly, this panel of mutations exhibits similar effects on the functional interaction of 14-3-3ζ with ExoS, a 14-3-3-dependent-ADP-riboseyltransferase, suggesting that these two associated proteins share a common structural determinant involving these charged residues.

To test whether residues in the hydrophobic side of the groove are involved in ligand binding, we have now individually replaced these residues with a negatively charged Asp, generating 14-3-3ζ with the point mutations, L172D, V176D, L216D, L220D, and L227D. We report here that L172D, V176D, and L220D drastically disrupted the interaction of 14-3-3ζ with Raf-1 kinase and that L172D and L227D showed moderate effects. These hydrophobic residues appear to be required for the binding of 14-3-3ζ to the recognition sites of Raf-1 under both activated and resting status. Importantly, the same mutations disrupted the association of 14-3-3ζ with two other regulatory proteins, Cbl and c-Ber, suggesting that the hydrophobic residues of the groove may be part of a binding site shared by multiple target proteins.

MATERIALS AND METHODS

Strains and Plasmids—Escherichia coli strains were grown routinely at 37 °C in LB liquid medium or agar plates (15 g/liter). Strain BL21(DE3) was used for protein expression. Saccharomyces cerevisiae strains were grown at 30 °C in synthetic medium supplemented with relevant amino acids and 2% glucose or galactose as a carbon source (33). Strain EGY48 (MATa trp1 ura3 his3 LEU2::LexAop6-LEU2) was used as the host strain in a yeast two-hybrid assay (33, 34). pHAPE625 (31) carrying the 14-3-3ζ gene was used as a template for mutagenesis. The 14-3-3ζ DNA fragments carrying various mutations were subcloned into pEG405 and pEG202 vectors (34) using the EcoRI-XhoI sites and used in a yeast two-hybrid system. For the purpose of protein expression in E. coli, the 14-3-3ζ gene and its mutant derivatives were subcloned from pHAPE625 into the pET-15b expression vector (Novagen, Madison, WI) using the NdeI-EcoRI sites.

Cell Culture and Stimulation—Human Jurkat T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum to a density of 1 × 106 cells/ml essentially as described (24). Prior to PMA treatment, cells were collected by centrifugation (600 × g, 5 min), washed with phosphate-buffered saline, resuspended in RPMI 1640 medium without fetal calf serum, and incubated at 37 °C for 12 h for starvation. Starved cells were collected and resuspended in RPMI 1640 medium without serum to a density of 2 × 106 cells/ml. After equilibration at 37 °C for 5 min, cells were activated with PMA in MeSO (50 ng/ml final) for 15 min. Stimulation was terminated by adding an equal volume of ice cold 2× Nonidet P-40 lysis buffer (2% Nonidet P-40, 40 mM Tris-HCl, pH 7.5, 10 mM EDTA, 300 mM NaCl, 10 mM NaF, 10 mM Na3PO4, 4 mM Na2VO4, 20 μg/ml each of aprotinin and leupeptin), and cells were lysed for 10 min on ice. The insoluble cell debris was removed by centrifugation (13,000 × g for 10 min) at 4 °C.

Site-Directed Mutagenesis—The 14-3-3ζ gene was achieved with the unique site elimination method (35) using double-stranded plasmid as a starting template. Two oligonucleotide primers were used: a mutagenic primer containing the desired mutation (see below) and a selection primer containing a mutation that eliminated the unique SacI site and introduced an EcoRV site (underlined) in the mutagenesis template pHAPE625 (selection primer: 5′-AAATGCTTCATGTTATCTGCTGAAAAGGAGGAG-3′). The incorporation of the mutagenic primers was screened by reverse enzyme digestion and verified by sequencing the entire 14-3-3ζ gene. The mutagenic primers used are listed below (mutations generated are underlined and the restriction sites introduced for screening purpose are in lowercase): 1) 5′-GACTGGGgtcagcGATAATCTTCGCTTG-3′ (Leu-172 to Asp, NheI site introduced), 2) 5′-CCTCTTCGACTTCTATTATGagatctGAACTG-3′ (Val-176 to Asp, Rf/I site introduced), 3) 5′-AAAGACgatattAGAATCGATGATAATCGATTTGTA-3′ (Leu-216 to Asp, SacI site introduced), 4) 5′-CTTATAGTGCAAGATCGATTTggcgAAAAGGAGGAG-3′ (Leu-220 to Asp, NruI site introduced), and 5) 5′-GATAACCTGCAGACTGAGtcgtggcGATAACCTGACG-3′ (Leu-227 to Asp, AarII site introduced).

Yeast Two-Hybrid Interaction Trap Assay—A modified version of the yeast two-hybrid system (36), the interaction trap (34), was used for studying 14-3-3ζ-Raf-1 interactions in vivo. S. cerevisiae strain EGY48 was used as a host strain for all interaction assays. Both pSH18-34 and pJK103 were used as reporters (33). pSH18-34 contains a GAL1-lacZ gene under control of eight high affinity ColE1 LexA operators, whereas pJK103 reporter has two ColE1 LexA operators. pEG202 derivatives were constructed to encode LexA-Raf-1 (full length), LexA-Raf (amino acids 321–648), LexA-14-3-3ζ (amino acids 1–320), LexA-14-3-3ζ-P62 (amino acids 269–648) and LexA-14-3-3ζ-mutant fusion proteins (31). pG4-5 derivatives were generated to encode B42-14-3-3ζ WT and mutant fusion proteins. For the protein-protein interaction analysis, EGY48 harboring a lacZ reporter was co-transformed with the pEG202 and pG4-5 derivative plasmids by the lithium acetate method (33). Transformants were selected by complementation of auxotrophs by ura3, his3 or trp1 markers on plates of synthetic medium supplemented with 2% glucose.

Assay for β-Galactosidase Activity—For the X-gal plate assay, yeast transformants were patched onto synthetic medium plates containing 2% galactose, 1% raffinose, and 20 μg/ml X-gal (Gold Biotechnology, Inc., St. Louis, MO) to induce and detect the expression of the lacZ reporter gene. The color development from positive or negative interactions on X-gal plates was recorded after 24 h of incubation at 30 °C. For the quantitative liquid assay, yeast transformants were first grown in selective medium with 2% glucose as carbon source to Aoos ~ 1.0 and then transferred to induction medium with 2% galactose and 1% raffinose as carbon source essentially as described (31). β-Galactosidase activity quantification, chlorophenyl-red-β-D-galactopyranoside was used as a chromogenic substrate. β-Galactosidase activities were calculated as described previously (31). Three independent colonies were used for each activity determination.

Protein Expression and Purification—14-3-3ζ WT and mutant proteins were expressed in E. coli BL21(DE3). Expression of hexahistidine-tagged 14-3-3ζ proteins was induced with isopropyl-1-thio-β-D-galactopyranoside as described previously (26). Crude extracts were prepared by sonication on ice followed by centrifugation (15,000 × g, 10 min). The
supernatants were used as the source of hexahistidine-tagged 14-3-3ζ proteins and directly used for the solid phase binding assays as described below. For the purpose of protein purification, the crude extracts were subjected to Ni²⁺-chelating chromatography. 14-3-3ζ proteins were purified essentially as described (26). Protein concentration was determined using the Comassie blue dye-based Bio-Rad Protein Assay kit with bovine serum albumin as a standard.

**Gel Filtration Chromatography**—For monitoring the dimeric status of 14-3-3ζ proteins, 1 μg of each purified WT or mutant 14-3-3ζ protein was applied through a 25-μl injection loop to a Superdex 75 column (HR 10/30, Amersham Pharmacia Biotech) equilibrated with HEPES buffer (10 mM, pH 7.3, 150 mM NaCl). The chromatography was performed on an Amersham Pharmacia Biotech FPLC system at 4 °C with a flow rate of 0.5 ml/min. Elution positions of dimeric and monomeric 14-3-3ζ proteins were determined by using a panel of non-denatured protein molecular weight standards (Sigma) and confirmed by the elution position of denatured, monomeric 14-3-3ζ.

**Solid Phase Binding Experiments**—Ni²⁺-chelated Sepharose 6B beads (Novagen) were incubated with E. coli crude extracts containing hexahistidine-tagged 14-3-3ζ proteins to generate beads coated with 14-3-3ζ for the subsequent binding assays. Two types of binding assays were employed. One used a radiolabeled binding partner generated by the Tnt in vitro transcription/translation system (Promega, Madison, WI). The other used total cell lysates from cultured Jurkat T cells. For the latter assay, 14-3-3ζ coated beads (~5 μg of protein each) were incubated with 5 μl of Tnt reaction mix containing [35S]methionine-labeled Raf-1. The 14-3-3ζ-Raf-1 complex was washed three times with Nonidet P-40 buffer (31). The protein complexes formed in the binding assay were eluted from beads by boiling (3 min) in 10 μl of SDS sample buffer and resolved by SDS-PAGE (12.5%). The presence of the radiolabeled Raf-1 protein in the 14-3-3ζ complex was visualized using a PhosphorImager (Molecular Dynamics, Inc.). For the Jurkat T cell lysate binding assay, 200 μl each of the Jurkat T cell lysates (from 2 x 10⁶ cells) was incubated individually with WT or mutant 14-3-3ζ-coated beads that contained about 5 μg of 14-3-3ζ protein. Suspeensions were incubated at 4 °C with mild rotation for 1 h and washed three times with Nonidet P-40 buffer. The beads were boiled for 3 min in 10 μl of SDS sample buffer. After centrifugation, the supernatant was subjected to SDS-PAGE and immunoblotting. Proteins that were complexed with 14-3-3ζ were visualized with corresponding antibodies using ECL (Amersham Pharmacia Biotech).

**Partial Proteolytic Digestion**—2.5 μg of each of the purified 14-3-3ζ WT and mutant proteins was incubated with 0.5 unit of chymotrypsin or Pronase in a total volume of 10 μl at 37 °C. After digestion, the products were subjected to SDS-PAGE (15%) followed by silver staining (Bio-Rad). The digestion patterns of mutant proteins were compared with those of the WT.

**CD Spectroscopy**—The far-UV CD spectra of 14-3-3ζ WT and mutant proteins were recorded on a Jasco-600 Spectropolarimeter as described (31). Results are expressed as the mean residue molar ellipticity ([θ], deg cm² dmol⁻¹) calculated from the equation: [θ] = ([θ]o × MRW / (10 × T × C)), where [θ]o is the observed ellipticity in millidegrees, MRW is the mean residue molecular weight (114 for 14-3-3ζ), L is the optical path length in cm (0.1 cm), and C is the final protein concentration in mg/ml. Data were collected from 250 to 200 nm wavelength at 0.2-nm intervals. The protein concentration was set at 0.4 mg/ml in phosphate-buffered saline, and the final far-UV CD spectra were averaged over four independent scans.

**SDS-PAGE and Immunoblotting**—SDS-PAGE and the enzyme-linked immunoblotting procedures were performed essentially as described (31). Anti-HA monoclonal antibody from mouse (HA.11; BAbCO, Berkeley, CA) was used to detect 14-3-3ζ proteins fused with an influenza virus HA epitope in the pJG4-5 vectors in the yeast two-hybrid system. Raf-1, Cbl, and c-Bcr were detected by polyclonal anti-Raf-1, anti-Cbl (both from Santa Cruz Biotechnologies, Santa Cruz, CA), and anti-Bcr antibodies (generously provided by Dr. Ann Marie Pendergast). Corresponding secondary antibodies were used against each primary antibody: horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham) for anti-HA antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for anti-Raf-1, anti-Cbl, and anti-Bcr antibodies. Cross-reacting materials were visualized using ECL.

**RESULTS**

**Conserved Hydrophobic Residues in Helices 7 and 9 are Implicated in Ligand Binding**—In the three-dimensional crystal structure of 14-3-3ζ, residues from helices 3, 5, 7, and 9 define a conserved amphipathic groove (30). On one side of the groove, residues such as Lys-49, Arg-56, and Arg-60 form a charged surface with their exposed side chains. On the other side, the side chains of Leu-172, Val-176, Leu-216, Leu-220, and Leu-227 from helices 7 and 9 project toward the inside of the groove forming a hydrophobic surface (Fig. 1). We proposed that this conserved, amphipathic groove may allow 14-3-3ζ to interact with a common structure on its diverse associated proteins (30). Consistent with this hypothesis, charge reversal mutations of charged residues in this groove (K49E, R56E and R60E) decrease the interaction of 14-3-3ζ with Raf-1 kinase and Eos8 (31). To further examine the contribution of the amphipathic groove to ligand binding, we proceeded to disrupt the hydrophobic side of the groove by site-directed mutagenesis and tested the effect of such alteration on the interaction of 14-3-3ζ with Raf-1 kinase.

**Mutations of Leu-172, Val-176, Leu-216, Leu-220, and Leu-227 Decrease the Binding of 14-3-3ζ to Raf-1 Kinase**—We replaced hydrophobic amino acids individually with a negatively charged residue, Asp, generating L172D, V176D, L216D, L220D, and L227D mutants. The effects of mutations in the hydrophobic surface on the binding of 14-3-3ζ to Raf-1 kinase were tested using two assays: a genetic two-hybrid assay in yeast and a solid phase binding assay in vitro. The yeast two-hybrid system provides a way of determining protein-protein interactions in an in vivo environment (36). It has been shown that in the two-hybrid system, the interaction of LexA-Raf-1 fusion with B42-14-3-3ζ fusion protein results in transcriptional activation of a lacZ reporter and, consequently, β-galactosidase activity that can be visualized using a chromo-

![FIG. 1. Atomic model (solid sphere representation) of the 14-3-3ζ monomer.](image-url)
Mutant 14-3-3 Proteins

Effect of mutations L172D, V176D, L216D, L220D, and L227D of 14-3-3ζ on its interaction with Raf-1 kinase in yeast. Yeast strain EGY48, harboring the reporter plasmid pSH18-34 and the expression vectors pEG202 (carrying LexA-Raf) and pJG4-5 (carrying B42-14-3-3ζ WT or mutant derivatives), were grown in synthetic medium with galactose. Cells were collected and used for both β-galactosidase liquid assay (upper panels) and immunoblotting analysis (lower panels), as described under “Materials and Methods.” The vertical bars represent units of β-galactosidase activity (n = 3). The lower panels show the relative levels of WT and mutant 14-3-3ζ proteins expressed in test strains. For these experiments, equivalent amounts of total cell protein were used in each lane. Anti-HA antibody was used to probe the B42-14-3-3ζ proteins, because an HA epitope was inserted between the B42 and 14-3-3ζ sequences in the pJG4-5 vectors. Control strains contained pEG202 (carrying the respective raf genes) in combination with plasmid pJG4-5.

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Mutant 14-3-3 Proteins

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mutations did not induce a global structural change of 14-3-3ζ protein. To ensure the validity of this assay, we have taken multiple time points and used two different concentrations of the enzyme (0.5 and 1.0 units). Similar results were obtained (data not shown). Furthermore, limited digestion by a different, nonspecific Pronase (0.5 unit for 30 min) also showed cleavage patterns of above mutants similar to that of the WT protein. Then, CD spectroscopy was used to assess the structural similarities between WT and mutant 14-3-3ζ proteins because it provides a sensitive measurement of protein secondary structural changes. Consistent with the three-dimensional crystal structure, which shows that α-helices are the major component of 14-3-3ζ proteins (30), far UV CD spectra of WT and mutant 14-3-3ζ proteins were characteristic of an α-helical structure, with ellipticity minima occurring at 208 and 222 nm (Fig. 5). The CD spectra of WT 14-3-3ζ and its mutant derivatives were essentially identical.

However, L172D protein was less stable than the WT. It was digested more rapidly than WT and other mutant proteins, showing decreased intensity of the large molecular weight band (Fig. 4). This result is most likely because of a conformational change of the L172D protein that exposes additional cleavage sites for chymotrypsin. Consistent with this, storage at 4 °C induced gradual degradation and aggregate formation of the L172D mutant protein, and such samples showed altered CD spectra, suggesting a reduced stability compared with the WT. Furthermore, an alternative approach, gel filtration chromatography, was employed to test dimer formation of WT and its mutant derivatives (Fig. 6). On a calibrated Superdex 75 column (HR 10/30), WT 14-3-3ζ eluted as a single peak with an apparent molecular mass of about 60 kDa, suggesting a dimeric form. With the exception of L172D, all individual mutant proteins eluted as dimers as did WT. L172D eluted as a broad shifted peak, which may represent partially degraded proteins. Therefore, V176D, L216D, L220D, and L227D mutant proteins are not dimerization defective.
Mutant 14-3-3 Proteins

V176D, and L220D abolished the association of 14-3-3ζ with Raf-1 from unstimulated and stimulated cells, and L216D partially decreased the binding. The effect of L220D is not obvious in this assay. Interestingly, L220D, which drastically decreased the binding of 14-3-3ζ to Raf-1, was not a negative control for the 14-3-3ζ-Raf-1 association. This difference may suggest ligand-specific contacts with the hydrophobic residues in the amphipathic groove of 14-3-3ζ are required for binding to Raf-1 under both resting and activated states.

Mutations of the Hydrophobic Surface Residues Decrease the Binding of 14-3-3ζ to Cbl and c-Bcr—We reasoned that mutations that disrupted the binding of 14-3-3ζ to Raf-1 should also decrease its interaction with other ligands if this putative binding site is indeed shared by multiple partners. To test this model, we examined the effect of the above hydrophobic mutations on the interaction of 14-3-3ζ with two other proteins known to bind 14-3-3ζ, Cbl and c-Bcr.

Cbl is a 120-kDa protein-tyrosine kinase substrate in activated T cells (41, 42). It specifically binds 14-3-3ζ in a phosphorylation-dependent manner in Jurkat T cells when stimulated with either TCR/CD3 ligation or PMA (9). Mutational analysis of Cbl protein suggested a putative 14-3-3ζ-binding site that is distinct from the 14-3-3-binding motifs defined in Raf-1 (23, 24). Consistent with the previous report (9), we found that WT 14-3-3ζ only bound to Cbl protein from PMA-stimulated T cells. No detectable Cbl was complexed with immobilized 14-3-3ζ when cells were not treated with PMA (Fig. 7). Mutations L172D, V176D, and L220D abolished the interaction of 14-3-3ζ with stimulated Cbl, whereas L216D and L227D showed only a slight effect. Like WT 14-3-3ζ, none of the 14-3-3ζ mutant proteins were capable of binding to Cbl from resting cells. K49E, a negative control for the 14-3-3ζ-Raf-1 interaction, was also defective in Cbl binding. It appears that the hydrophobic residues in the amphipathic groove of 14-3-3ζ are not only important for interaction with Raf-1.

To further confirm that the hydrophobic residues form part of a general binding site shared by multiple ligands, we extended our observations to the c-Bcr kinase. c-Bcr was discovered initially as an essential part of a chimeric oncogene product, Bcr-Abl, in Philadelphia chromosome-positive human leukemias (43). c-Bcr specifically associates with 14-3-3ζ possibly through its Cys- and Ser/Thr-rich regions (8). In agreement with previous reports, we found that WT 14-3-3ζ was able to bind c-Bcr from Jurkat T cells (Fig. 8). The previously defined mutation K49E was used as a control, which significantly decreased the 14-3-3ζ-c-Bcr interaction (31). As in the case of Raf-1 and Cbl, L172D and V176D abolished the association of 14-3-3ζ with c-Bcr. The effect of L216D and L227D was not obvious in this assay. Interestingly, L220D, which drastically disrupted the interaction of 14-3-3ζ with Raf-1 and Cbl, showed only slight effect on the 14-3-3ζ-c-Bcr association. This difference may suggest ligand-specific contacts with the hydrophobic residues. In general, it is clear that mutations of the hydrophobic surface of the conserved amphipathic groove similarly disrupted the interactions of 14-3-3ζ to multiple targets, including Raf-1, Cbl, and c-Bcr, therefore this hydrophobic surface may define part of a general ligand-binding site in the 14-3-3ζ molecule.

**DISCUSSION**

14-3-3 proteins are involved in multiple signaling pathways and interact directly with a wide range of regulatory molecules such as Raf-1, Cbl, and c-Bcr. Our three-dimensional crystal structure of 14-3-3ζ suggests that a conserved amphipathic groove of the molecule may mediate its interaction with multiple ligands (30). Previously, we found that charge reversal mutations in this putative binding site (K49E, R56E, and R60E) impair the binding of 14-3-3ζ to Raf-1 kinase. We have
shown here that mutations that alter the hydrophobic surface of this conserved groove decreased the interaction of 14-3-3ζ to Raf-1 kinase and that this effect was not restricted to Raf-1. Specifically, mutations L172D, V176D, L216D, L220D, and L227D, to different extents, also decreased the binding of 14-3-3ζ to two other regulatory proteins, Cbl and c-Bcr. It appears that the hydrophobic side chains of the groove are as important as the charged residues for the association of 14-3-3ζ with multiple ligands. Together, the above data strongly support the model that 14-3-3-associated proteins share a common binding site on 14-3-3ζ involving an amphipathic groove.

A general mode of 14-3-3 protein-target interactions involves a phosphorylated Ser residue in the target proteins (21–23). Our mutational analysis together with the available structural and genetic data has suggested a possible mechanism by which the conserved amphipathic groove mediates the association of 14-3-3ζ with a phosphorylated ligand. As discussed above, mutations that alter the amphipathic property of the groove decreased the binding affinity of 14-3-3ζ to its associated ligand. In support of a direct role for the amphipathic groove in binding to a phosphorylated ligand, our crystallographic data show that a Ser-phosphorylated peptide derived from Raf-1 (LSSQKRRS'TpSTPNVHMV) binds in an extended conformation in the groove (48). A middle T antigen–based phosphoserine peptide is also found in this amphipathic groove (44), which lends further support to our model. In the 14-3-3ζ crystal structure, a sulfate ion was identified that binds in the groove, making contact with the Arg/Lys cluster including Lys-49, Arg-56, and Arg-60 (44). In our electron density map of the 14-3-3ζ peptide complex, there is a strong peak at the same position, suggesting that the phosphate moiety of the phosphoserine binds at this site. Val-176 is closest among those hydrophobic residues tested to the cluster of positive charges of 14-3-3ζ and is only 5 Å away from the phosphate group in our 14-3-3ζ peptide complex. Thus it may interact with the main chain of the peptide ligand or a hydrophobic side chain of the ligand. Not surprisingly, the V176D mutation exhibited the most potent effect on the ligand binding, and a preserved change at this site, V176A, was sufficient to disrupt the binding of 14-3-3ζ to Raf-1 kinase (data not shown).

In agreement with the above notion, alleles of BMHI (a 14-3-3 homologue) defective in Ras/mitogen-activated protein kinase signaling during pseudohypophyseal development in yeast were mapped to the putative ligand-binding groove as mutations G55D, A59T, and L232S (45). Gly-55 and Ala-59 of Bmh1p correspond to Gly-53 and Ser-57 in 14-3-3ζ, and L232S coincides with Leu-227 that we have analyzed here. In our structure, Gly-53 and Ser-57 are located inside of the groove, the side chain of Ser-57 points toward the basic cluster in the groove, and Leu-227 forms part of the hydrophobic surface (Fig. 1). Mutations at these residues are expected to have some effect on ligand binding if the amphipathic groove is involved in the protein–protein interactions. Indeed, G55D, A59T, and L232S of Bmh1p exhibit decreased affinities for Ste20, an upstream component of the Ras/mitogen-activated protein kinase signaling cascade in yeast (45).

14-3-3 proteins have also been shown to bind ligands in a phosphorylation-independent manner. For example, 14-3-3ζ is able to interact with Raf-CRD (25) and a bacterial ADP-riboseyltransferase (26). It appears that the unphosphorylated ExoS binds 14-3-3ζ at the amphipathic groove (31). Whether Raf-1 CRD and other unphosphorylated ligands bind 14-3-3ζ through the same structural determinant remains to be determined.

14-3-3 proteins may bind to distinct sites of Raf-1 depending on its activation status (27). To test whether there is any difference in structural requirements for Raf-1 binding, we probed the interaction of 14-3-3 mutants with Raf-1 from either resting Jurkat T cells or cells treated with PMA. As reported, 14-3-3ζ was able to bind full-length Raf-1 regardless of its activation status (4, 6, 24). PMA treatment induced the hyperphosphorylation of Raf-1 with no obvious alteration of 14-3-3 binding. Mutations of the hydrophobic residues in the binding groove decreased the binding of 14-3-3ζ to full-length Raf-1 kinase independent of its activation status, suggesting that the hydrophobic residues are necessary for the binding of 14-3-3ζ to Raf-1 under both states.

The disruption of 14-3-3ζ-Raf-1 interaction by the mutations V176D, L216D, L220D, and L227D cannot be explained by gross structural alteration, as evidenced by partial proteolysis and CD analyses. Importantly, these mutants are also capable of forming dimers in vivo and in vitro. The hydrophobic residues mutated in this study are all fully solvent-accessible, with the exception of Leu-172, which is partly buried and packs against the main chain of helix 9. This presumably explains the relative instability of this mutant. It remains unclear whether Leu-172 is directly involved in ligand binding. The effect of L216D and L227D on 14-3-3-Raf association was significant in the yeast two-hybrid assay (Fig. 2). However, these two mutations showed only a slight effect on 14-3-3-ligand interactions utilizing a solid phase binding assay, which may be because of the limited sensitivity of the in vitro binding assay for detecting minor changes in protein-protein interactions.

In summary, data presented here strongly support the model that the hydrophobic residues lying in the amphipathic groove of 14-3-3ζ constitute part of a general binding site for multiple 14-3-3-associated proteins. Because the ligand-binding mutants identified by our work are competent in dimer formation, it is possible that the expression of these mutant proteins in cells may exhibit dominant interfering effect by disrupting the normal function of endogenous 14-3-3 proteins through mutant-WT heterodimer formation. Use of such mutants is expected to shed light on how 14-3-3 proteins modulate cellular functions.

Acknowledgments—We thank Romesh Subramanian and Hillary Van Valkenburgh for technical assistance and Drs. Todd Milne (MIT) and Sims Kochi (VRD) for critical reading of the manuscript and helpful comments. We also thank members of our laboratory for valuable suggestions.

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