Identification of 14-3-3ζ as a Protein Kinase B/Akt Substrate

David W. Powell§, Madhavi J. Rane§, Qingdan Chen§, Saurabh Singh§,

and Kenneth R. McLeish§,§,•

Departments of Biochemistry and Molecular Biology§ and Medicine§, University of Louisville,

and The Veterans Affairs Medical Center•, Louisville, KY 40202

Correspondence should be addressed to:

Kenneth R. McLeish, M.D.
Molecular Signaling Group
Kidney Disease Program
615 South Preston Street
University of Louisville,
Louisville, KY 40202-1718
Telephone: 502-852-7194
Fax: 502-852-4384
E-mail: k.mcleish@louisville.edu
Abstract

Protein kinase B/Akt (PKB/Akt) is a member of the ACG kinase family, which also includes protein kinase C, that phosphorylates a number of 14-3-3 binding proteins. 14-3-3 protein regulation of protein kinase C activity is modulated by 14-3-3 phosphorylation. We examined the hypothesis that PKB/Akt interacts with and phosphorylates 14-3-3ζ, leading to modulation of dimerization. By GST pull-down, GST-Akt precipitated recombinant 14-3-3ζ and endogenous 14-3-3ζ from HEK293 cell lysates. Recombinant active PKB/Akt phosphorylated recombinant 14-3-3ζ in an in vitro kinase assay. Transfection of active PKB/Akt into HEK293 cells resulted in phosphorylation of 14-3-3ζ. Based on a motif search of 14-3-3ζ, a potential PKB/Akt phosphorylation site, Ser-58, was mutated to alanine. PKB/Akt was unable to phosphorylate this mutant protein. Incubation of 14-3-3ζ with recombinant active PKB/Akt resulted in phosphorylation of 455 of the protein, as determined by a pI shift on two dimensional electrophoresis, but 14-3-3ζ dimerization was not altered. These data indicate that PKB/Akt phosphorylates Ser-58 on 14-3-3ζ both in vitro and in intact cells. The functional relevance of this phosphorylation remains to be determined.
Introduction

Protein kinase B (PKB)/Akt is a member of the AGC family of serine/threonine kinases, named for the original members, protein kinase A, cGMP-dependent protein kinase, and protein kinase C. AGC kinases require phosphorylation of both a residue in the kinase domain (Thr308 in PKB/Akt) and in the regulatory C-terminal hydrophobic domain (Ser473 in PKB/Akt) for full kinase activity (1,2). PKB/Akt and most other members of the AGC family are activated by phosphatidylinositol 3-kinase-mediated activation of phosphatidylinositol-dependent kinase-1 (PDK1), which phosphorylates the kinase domain residue (1,2). Numerous substrates of PKB/Akt have been identified, including BAD, IKKα, Raf kinases, forkhead transcription factor family members (FKHR and FKHRL1), insulin receptor substrate-1 (IRS-1), glycogen synthase kinase-3 (GSK-3), apoptosis signal-regulating kinase 1 (ASK1), mammalian target of rapamycin (mTOR), rac1 and cdc42, p27 CDK inhibitor, and p21cip1/WAF1 (2-4). Functional responses regulated by these substrates of PKB/Akt include cell growth and proliferation, cell survival, angiogenesis, protein synthesis, glycogen synthesis, translocation of glucose transporters, and gene transcription (1-4).

The 14-3-3 proteins are a family of adaptor and scaffolding proteins that interact with more than 60 proteins and are expressed in all eukaryotic cells (5,6). Seven mammalian isoforms (β,ε,γ,η,σ,τ,ζ) with molecular masses of 28 to 33 kDa have been identified. These proteins form homo- and hetero-dimers that interact with phosphoserine binding domains (7). A number of PKB/Akt substrates bind to 14-3-3 proteins after phosphorylation, including BAD, FKHRL1, ASK1, p27 CDK inhibitor, and p21cip1/WAF1 (8-11). Other proteins that may be binding partners of both PKB/Akt and 14-3-3 proteins include Raf kinases, IRS-1, and Cbl (2,5). Montano reported that PKB/Akt and 14-3-3 share two amino acid sequences that mediate
protein-protein interactions (12). Thus, there is significant cooperativity between PKB/Akt and 14-3-3 proteins in the regulation of some cellular functions.

14-3-3 proteins bind to some members of the ACG kinase family, including protein kinase C, and regulate their activation (13). Additionally, phosphorylation of 14-3-3 proteins impairs their interaction with protein kinase C (14). Three phosphorylation sites on 14-3-3ζ, Ser-58, Ser-184, and Thr-232, have been identified (5). Sphingosine-dependent protein kinase (SDK-1) phosphorylates Ser-58 and casein kinase I phosphorylates Thr-232, although the role of these phosphorylations in the regulation of 14-3-3 function is unknown (15,16). A computer-based (Scansite.mit.edu) evaluation of PKB/Akt phosphorylation motifs on 14-3-3 identified two low stringency sites, Ser-58 and Ser-63 (17). These findings suggest the possibility that PKB/Akt might phosphorylate 14-3-3 proteins and regulate a function common to both proteins. The present study was initiated to determine if PKB/Akt interacts with and phosphorylates 14-3-3ζ and if phosphorylation by PKB/Akt regulates 14-3-3ζ dimerization.
Materials and Methods

Expression Vectors. pUSE-myc-constitutive active Akt and pUSE-Akt were from Upstate Biotechnology, Lake Placid, NY. PGEX-2T-14-3-3ζ and pcDNA3.1-HA-14-3-3ζ were obtained from Dr. Thierry Dubois, University of Edinburgh, UK. 14-3-3ζ was subcloned from PGEX-2T-14-3-3ζ into pRSET using BamHI and EcoRI, and pRSET-14-3-3ζ was used for bacterial expression and purification of recombinant 14-3-3ζ.

Ser-58 on pRSET 14-3-3ζ was mutated to alanine with Clontech tranformer site-directed mutagenesis kit, using 5’-CCGTAGGTCAGGGTGGAGGGTGG-3’ as the mutation primer and 5’-CAGCAGGTGGCTCGGAATACAGAGA-3’ as the selection primer. The appropriate mutation was verified by DNA sequencing. His-tagged recombinant 14-3-3ζ (S58A) was bacterially expressed and isolated by nickel chromatography.

Akt kinase assay. Recombinant active Akt (400 ng) (Upstate Biotechnology) was incubated with 10 μCi [γ-32P]ATP (167 TBq/mmol, ICN Biomedicals, Inc., Irvine, CA) and 1 μg recombinant 14-3-3ζ in 30 μl kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiotreitol, and 0.1 mM NaVO3). Reactions were incubated at 30°C for 2 hours and terminated by addition of Laemmli SDS sample dilution buffer. Proteins were separated by 10% SDS-PAGE, and phosphorylation was visualized by autoradiography.

GST-pull down. Ten μl glutathione agarose beads coupled to GST or GST-Akt (Upstate Biotechnology) were incubated with 250 ng recombinant 14-3-3ζ in 50 μl kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiotreitol, and 0.1 mM NaVO3) or with HEK293 cell lysate (800 μg protein) overnight at 4°C. HEK293 lysates were prepared by
suspending 2 x 10^7 cells in 200 μl lysis buffer containing 20 mM Tris, pH 7.4, 1% Triton x-100, 0.5% NP-40, 150 mM NaCl, 25 mM MgCl_2, 20 mM NaF, 0.2 mM NaVO_3, 1 mM EDTA, 1 mM EGTA, 5 mM PMSF, and 10% glycerol. Following incubation, beads were washed twice with lysis buffer. Proteins were eluted with Laemmli SDS sample dilution buffer, separated by 10% SDS-PAGE, and immunoblotted with anti-14-3-3ζ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

In situ phosphorylation. HEK 293 cells (60% confluent) were incubated with 6:1 lipofectamine:plasmid in OPTI medium (Gibco, Grand Island, NY) at 37°C for 6 hours. Medium was then replaced with DMEM, 10% fetal bovine serum and cells were incubated at 37°C overnight. HEK 293 cells were transfected with pcDNA3.1-HA-14-3-3ζ, pUSE-myc-constitutive active-Akt, or co-transfected with pcDNA3.1-HA-14-3-3ζ and pUSE-myc-constitutive active-Akt. Following overnight cultivation, the medium was replaced with 3 ml phosphate free MEM (Gibco) and incubated at 37°C for 1 hour. The medium was then replaced with 1 ml MEM containing 1 mCi [32P]-orthophosphate and incubated at 37°C for 4 hour. Cells were then lysed with 520 μl lysis buffer containing 20 mM Tris, pH 7.4, 1% Triton x-100, 0.5% NP-40, 150 mM NaCl, 25 mM MgCl_2, 20 mM NaF, 0.2 mM NaVO_3, 1 mM EDTA, 1 mM EGTA, 5 mM PMSF, and 10% glycerol. Twenty μl aliquots were taken to confirm transfection by immunoblot analysis for myc and HA. Lysates (500 μl) were incubated with 4 μg monoclonal anti-HA (Covance, Berkeley, CA) overnight at 4°C, followed by 2-hr incubation with 30 μl (1:1 slurry in PBS) protein A sepharose beads. Beads were washed twice with lysis buffer and proteins were eluted with Laemmli SDS sample dilution buffer and separated by 10% SDS-PAGE. Incorporation of [P^{32}] was visualized by autoradiography. Identification of
phosphorylated proteins was determined by MALDI-MS and peptide mass fingerprinting, as previously described (18).

14-3-3 Dimerization. Recombinant 14-3-3ζ (2 µg) was incubated alone, with recombinant active Akt (400 ng) (Upstate Biotechnology) and no ATP, or with recombinant active Akt and 1 mM ATP in 30 µl kinase buffer (25 mM HEPES, 25 mM β-glycerophosphate, 25 mM MgCl₂, 25 mM MnCl₂, 2 mM dithiotreitol, and 0.1 mM NaVO₃) for 2 hours at room temperature. Following incubations, proteins were separated by 10% non-denaturing PAGE followed by protein staining with coomassie blue or by two dimensional gel electrophoresis using pI 3-7 immobilized pH gradient strips for isoelectric focusing and 10% Duracryl gels (Genomic Solutions, Ann Arbor, MI) in the second dimension. Proteins were transferred to nitrocellulose and immunoblotted for 14-3-3ζ.
Results and Discussion

The ability of PKB/Akt to directly interact with 14-3-3ζ was examined using GST pull-down assays. GST-Akt coupled glutathione agarose was incubated with recombinant 14-3-3ζ or HEK293 lysate, followed by immunoblot analysis for 14-3-3ζ. Figure 1A shows that recombinant 14-3-3ζ was precipitated by GST-Akt-agarose, but not by GST-agarose. Similarly, GST-Akt-agarose, but not GST-agarose, precipitated endogenous 14-3-3ζ from HEK293 cell lysate (Figure 1B). To determine if PKB/Akt phosphorylated 14-3-3ζ, recombinant active PKB/Akt was incubated with recombinant 14-3-3ζ in an in vitro kinase assay. Figure 2 shows that PKB/Akt phosphorylated 14-3-3ζ, as well as undergoing auto-phosphorylation. No phosphorylation of 14-3-3ζ was seen in the absence of recombinant active PKB/Akt. These data demonstrate that PKB/Akt and 14-3-3ζ directly interact in vitro, and this interaction is associated with PKB/Akt phosphorylation of 14-3-3ζ.

The ability of PKB/Akt to phosphorylate 14-3-3ζ in intact cells was examined using HEK293 cells. Cells were transfected with constitutively active PKB/Akt and/or HA-tagged 14-3-3ζ. After 24 hr cells were loaded with [32P]-orthophosphate, lysed, and 14-3-3ζ immunoprecipitated with anti-HA antibody. Precipitated proteins were separated by SDS-PAGE and phosphorylated proteins were visualized by autoradiography. Figure 3A shows phosphorylation of a 30 kDa protein that was confirmed to be 14-3-3ζ by MALDI-MS. These findings indicate that PKB/Akt is capable of interacting with and phosphorylating 14-3-3ζ in intact cells.

Our data show that PKB/Akt interacts with and phosphorylates 14-3-3 proteins in vitro and in intact cells, as described previously for protein kinase C (13,14,19-22). 14-3-3τ and β,
but not 14-3-3ζ, couple protein kinase Cα to Raf-1 and facilitate Raf-1 activation (14). 14-3-3γ interacts with and is phosphorylated by multiple protein kinase C isoforms (19). Interaction with various 14-3-3 isoforms has been reported to stimulate (21) and to inhibit (13,20) protein kinase C activity and to link protein kinase C with Raf-1 kinase (14). Phosphorylation of Ser-184 increases 14-3-3 inhibition of protein kinase C in vitro (22). Other kinases reported to phosphorylate 14-3-3 isoforms include casein kinase I and SDK-1 (15,16).

Three phosphorylation sites have been identified on 14-3-3 proteins, Ser-58, Ser-184, and Thr-232 (5). As Ser-58 was recognized as a low stringency PKB/Akt phosphorylation (2.703%) site by Scansite (17), we created a mutant 14-3-3ζ in which alanine was substituted for Ser-58 (14-3-3ζ S58A). The ability of recombinant active PKB/Akt to phosphorylate this mutant protein was tested in an in vitro kinase assay. Figure 4 shows that PKB/Akt phosphorylated wild type recombinant 14-3-3ζ, while phosphorylation of 14-3-3ζ (S58A) was markedly inhibited. These data indicate that, like SDK-1, PKB/Akt phosphorylates Ser-58 on 14-3-3ζ.

Based on the three-dimensional structure of 14-3-3, phosphorylation of Ser-58 was proposed to regulate dimer formation (5,15). The effect of 14-3-3ζ phosphorylation by PKB/Akt on dimerization was examined in an in vitro assay. Recombinant 14-3-3ζ was incubated in the presence or absence of active recombinant PKB/Akt. Proteins were then separated on non-denaturing SDS-PAGE and 14-3-3ζ monomers and dimers identified by protein staining. Figure 5A shows that the proportion of 14-3-3ζ in the dimeric form was not altered by phosphorylation by PKB/Akt. In parallel, 14-3-3ζ incubated in the presence or absence of active PKB/Akt was subjected to two-dimensional gel electrophoresis, followed by immunoblot analysis for 14-3-3ζ (figure 5B). The immunoblot demonstrated a pI shift consistent with phosphorylation of 14-3-3ζ on only one amino acid. The percentage of 14-3-3ζ phosphorylated by PKB/Akt was estimated
to be 45%, based on densitometry of protein at the two pIs. Although our data do not reveal the functional significance of PKB/Akt phosphorylation of Ser-58 on 14-3-3ζ, recent studies indicate that Arg-56 forms a salt bridge with phosphoserine on 14-3-3 ligands, and that Ser-60 plays a minor role in phosphoserine binding (7,23). Thus, phosphorylation at Ser-58 may regulate 14-3-3 binding to phosphoserine-containing ligands, rather than to regulate dimerization.

In summary, our results indicate that PKB/Akt should be included with protein kinase C as another AGC kinase that phosphorylates 14-3-3 proteins. We determined that PKB/Akt directly interacts with 14-3-3ζ and phosphorylates 14-3-3ζ on Ser-58 both in vitro and in transfected HEK293 cells. Although 14-3-3ζ phosphorylation on Ser-58 has been proposed to regulate 14-3-3 dimerization, we could not detect an effect on this function. The crucial role of 14-3-3 binding to PKB/Akt substrates, however, suggests that PKB/Akt phosphorylation of 14-3-3 provides another level of regulation of PKB/Akt signaling.
Abbreviations:

Protein kinase B/Akt, PKB/Akt; phosphatidylinositol-dependent kinase-1, PDK1; insulin receptor substrate-1, IRS-1; glycogen synthase kinase-3, GSK-3; apoptosis signal-regulating kinase 1, ASK1; mammalian target of rapamycin, mTOR; sphingosine-dependent protein kinase, SDK-1; matrix assisted laser desorption ionization-mass spectrometry, MALDI-MS.
Figure legends:

**Figure 1.** *PKB/Akt and 14-3-3ζ interaction.* Panel A shows a GST pull-down in which recombinant 14-3-3ζ was incubated with glutathione-agarose coupled to GST or GST-Akt. Panel B shows a GST pull-down in which 293 lysate was incubated with glutathione-agarose coupled to GST or GST-Akt. Precipitated proteins were separated by SDS-PAGE, and 14-3-3ζ was detected by immunoblot analysis. In each case, 14-3-3ζ precipitated with GST-Akt agarose, but not with GST-agarose.

**Figure 2.** *In vitro PKB/Akt phosphorylation of 14-3-3ζ.* Recombinant active PKB/Akt, recombinant 14-3-3ζ, or recombinant active PKB/Akt and 14-3-3ζ were incubated in the presence of [32P]ATP. Proteins were separated by SDS-PAGE and autoradiography performed. The autoradiograph shows that Akt undergoes autophosphorylation and Akt phosphorylates 14-3-3ζ.

**Figure 3.** *PKB/Akt phosphorylation of 14-3-3ζ in HEK293 cells.* Panel A. HEK293 cells were transfected with HA-14-3-3ζ, HA-14-3-3ζ and myc-active PKB/Akt, or myc-active PKB/Akt alone. After 24 hr cells were loaded with [32P]orthophosphate, and 14-3-3ζ was immunoprecipitated by anti-HA antibody. Precipitated proteins were separated by SDS-PAGE, and phosphorylation of 14-3-3ζ was detected by autoradiography. Panel B. Expression of HA-14-3-3ζ and myc-active PKB/Akt by transfected HEK293 cells was confirmed by immunoblot (IB) analysis with anti-c-myc and anti-HA antibodies.
**Figure 4.** *PKB/Akt phosphorylates 14-3-3ζ at serine-58.* To determine if PKB/Akt phosphorylates 14-3-3ζ at Ser-58, recombinant active PKB/Akt was incubated with [³²P]ATP and recombinant 14-3-3ζ WT or 14-3-3ζ(S58A). Following kinase reactions, proteins were separated by SDS-PAGE and phosphorylation visualized by autoradiography. To demonstrate equal loading of 14-3-3 proteins, gels were also stained with coomassie blue.

**Figure 5.** *Effect of PKB/Akt phosphorylation on 14-3-3ζ dimer formation.*

Panel A. Recombinant 14-3-3ζ was incubated in the presence and absence of recombinant active PKB/Akt and ATP or recombinant active PKB/Akt in the absence of ATP. Following incubation for 2 hr, proteins were separated by 10% non-denaturing PAGE and Coomassie blue stained. The figure shows that incubation with PKB/Akt and ATP does not alter the proportion of dimeric 14-3-3ζ. Panel B. 14-3-3ζ incubated in the presence of absence of recombinant active PKB/Akt and ATP was separated by pH 4-7 isoelectric focusing followed by 10% SDS-PAGE. 14-3-3ζ was detected by immunoblot analysis. The immunoblots demonstrate a pI shift in 14-3-3ζ consistent with phosphorylation. The percent 14-3-3ζ phosphorylated was 45%, based on densitometry of the two forms of 14-3-3.
References

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Figure 1

A

GST  GST-Akt  GST  GST-Akt

25 kDa  14-3-3ζ IB

14-3-3ζ

B

GST  GST-Akt  GST  GST-Akt  293 Lysate

25 kDa  14-3-3ζ IB

293 Lysate
Figure 2

Active Akt  +  +  +
14-3-3ζ  +  +  +

50 kDa
Akt Auto-Phosphorylation

25 kDa
14-3-3ζ
Figure 4

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<tr>
<td>14-3-3ζ (S58A)</td>
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25 kDa

Autoradiograph

Coomassie Stain
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J. Biol. Chem. published online April 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203167200

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