Regulation of the Pro-apoptotic Scaffolding Protein POSH by Akt*

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POSH (Plenty of SH3 domains) binds to activated Rac and promotes apoptosis by acting as a scaffold to assemble a signal transduction pathway leading from Rac to JNK activation. Over-expression of POSH induces apoptosis in a variety of cell types, but apoptosis can be prevented by co-expressing the pro-survival protein kinase Akt. We report here that POSH is a direct substrate for phosphorylation by Akt in vivo and in vitro, and we identify a major site of Akt phosphorylation as serine 304 of POSH, which lies within the Rac-binding domain. We further show that phosphorylation of POSH results in a decreased ability to bind activated Rac, as does phosphomimetic S304D and S304E mutation of POSH. S304D mutant POSH also shows a strongly reduced ability to induce apoptosis. These findings identify a novel mechanism by which Akt promotes cell survival.

Another potential regulator of the POSH-JIP apoptotic complex appears to be the pro-survival kinase Akt, also known as protein kinase B. Three closely related Akt genes exist (Akt1–3) that have both significant functional overlap as well as specific unique functions (10, 11). Akt activation can protect cells from a variety of pro-apoptotic signals, and Akt can phosphorylate an increasing number of intracellular targets (12–22). Recently a direct binding interaction between POSH and Akt2 was described, in which binding of Akt2 to POSH prevents MLK3 binding and thereby inhibits PJAC assembly and apoptotic signaling by JNK. This binding interaction was found to be specific to Akt2; Akt1 showed no such binding to POSH (23). However, despite this isoform-specific interaction of Akt2 and POSH, activated Akt1 is fully able to protect cells from apoptosis induced by overexpression of POSH (1, 2). Thus, the precise physiologic importance of this isoform-specific interaction is unclear. It is also unclear whether Akt in general opposes POSH-induced apoptosis at the level of the PJAC complex or at more downstream sites. Furthermore, it has yet to be addressed whether Akt might regulate PJAC function by directly phosphorylating POSH and, if so, how this might affect POSH function. These questions are the subject of this study.

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† This paper is dedicated to the memory of Phil Ryan who died unexpectedly while this manuscript was under revision.

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The abbreviations used are: POSH, plenty of SH3 domains; SH, Src homology; MAPKK, mitogen-activated protein kinase kinase; MKK or MAPK; mitogen-activated protein kinase kinase; JNKS, c-Jun N-terminal kinases; MAPKS, mitogen-activated protein kinase; PJAC, POSH-JIP apoptotic complex; GST, glutathione S-transferase; GFP, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; MS, mass spectrometry; PAS, phospho-Akt-substrate antibody; DTT, dithiothreitol; BSA, bovine serum albumin.
**Phosphorylation of POSH by Akt**

**MATERIALS AND METHODS**

**Cell Culture**—HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. MDA-MB-231 cells were grown in the same media, additionally supplemented with 1 mM l-glutamine, minimum Eagle’s medium nonessential amino acids, and 10 ng/ml insulin. Fetal bovine serum was obtained from HyClone (Logan, UT). All other media components were obtained from Invitrogen.

**DNA Constructs and GST-POSH Fusion Proteins**—Human POSH cDNA was cloned by RT-PCR as follows. RNA was isolated from HEK293 cells using Trizol, as described by the manufacturer (Invitrogen), and used to prepare first strand cDNA using murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences) with oligo(dT) as primer. This cDNA was then used as template in two different PCRs to separately amplify the 5’- and 3’-halves of POSH, which were subsequently reassembled using the internal BamHI site. The 5’-half of POSH was amplified by PCR using the primers POSH-5’-R1 (GGCG GAA TTC ACT AGT ACC ATG GAT GAA TCA GCC TTG) and POSH-(1303–1326) (CCG TAA ATG TGC TTA CTG GTC AGT) and POSH-5’-R2 (GGG ATA CTC GCG GAT GCT GGC CGA) and myr-Akt2-HA (GAT TCT TCT AAG GCC ACC AGG CCC ATG GCA) and POSH-myc-Not (GCG TGC GGC CGC TCA CAG GTCT CTC CTG GTCT AGT) to generate a product extending to include the remaining coding sequence, followed by an engineered Myc epitope tag, and a NotI site. The two POSH fragments were then subcloned and reassembled to generate a full-length Myc-tagged cDNA. The resulting cDNA was then used as template in two different PCRs to separately amplify the 5’- and 3’-halves of POSH, which were subsequently reassembled using the internal BamHI site. The 5’-half of POSH was amplified using POSH-5’- (1273–1296) (ATG GGA CCC AGG CCC ATG GCA) and POSH-myc-Not (GGCG TGC GCC CGC TCA CAG GTCT CTC CTG GTCT AGT) to generate a product containing the endogenous BamHI site at its 5’-end and including the remaining coding sequence, followed by an engineered Myc epitope tag, and a NotI site. The two POSH fragments were then subcloned and reassembled to generate a full-length Myc-tagged cDNA. The resulting cDNA was entirely sequenced and confirmed to be in agreement with GenBank™ sequences for human POSH.

To generate GST-POSH fusion proteins in mammalian cells, the Myc-tagged POSH cDNA described above was subcloned into the vector pEBG-SrfI (a generous gift from Yusen Liu (24)), using Spel and NotI sites, to generate pEBG-POSH. To generate GST-POSH fusion proteins in *Escherichia coli*, the C-terminal Myc epitope tag was replaced with a 6-histidine tag using the primer POSH-3’-6His (GG CGC AGC GGC CGC TCA ATG ATG ATG ATG CGT CCA TCT ATT TTC AAC AAA GCT), and the fragment was subcloned into pGEX4T-1 (GE Healthcare) using EcoRI and XhoI or NotI sites, and the resulting GST-POSH fusion protein, were employed and gave similar results. The concentration of each DNA was 0.25 μg/μl. Green fluorescent protein (GFP)-positive cells were counted 4 h post-injection. 18 h following injection GFP-positive cells were again examined, assessed for viability/apoptotic changes, and quantitated. Cells were visualized using an Axiovert 200 MOT microscope (Carl Zeiss, Inc., Thornwood, NY). Fluorescence images were captured using a Hamamatsu ORCA ER CCD camera run by Openlab (Improvement, Lexington, MA).

**Transfections and Western Immunoblotting**—Transfections were performed by the calcium phosphate method, as described by Xu and Greene (26), with HEK293T cells plated at 30,000/cm² in 10-cm dishes 20–24 h prior to transfection, and fresh growth medium was added 3–4 h before transfection. Transfected cells were lysed 18–24 h later in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 50 mM NaF, 2 mM EDTA, 1 mM Na₂VO₄, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% SDS)

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supplemented with protease inhibitors (catalog number 1 697 498, Roche Applied Science). Lysates were clarified by centrifugation for 30 min at 13,000 × g at 4 °C, and protein concentrations were determined by the Bradford assay (Pierce). 1 mg of lysate protein was incubated with glutathione-Sepharose beads in binding buffer (50 mM NaCl, 50 mM NaF, 10 mM Tris, pH 7.4, 5 mM EDTA, 1 mM Na3VO4) overnight at 4 °C with rocking to recover GST-POSH. The beads were pelleted and washed three times with binding buffer. The proteins were then solubilized in SDS sample buffer and separated by SDS-PAGE, followed by transfer to Immobilon-P (Millipore, Bedford, MA) for immunoblotting. Antibodies used in immunoblots are as follows: anti-phospho-Akt substrate antibody (PAS) was obtained from Cell Signaling Technologies, anti-GST antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal anti-Rac antibody was from Sigma, and anti-POSH antibody was a generous gift of Dr. Yuval Reiss (Proteologics, Rehovot, Israel). Horseradish peroxidase-labeled secondary antibodies were obtained from Bio-Rad and visualized by chemiluminescence (Western Lightning, PerkinElmer Life Sciences).

In Vitro Kinase Reactions—1 μg of purified GST or GST-POSH protein was incubated with 20 ng of active Akt1 (Upstate catalog number 14-276, Millipore) or Akt2 (Upstate catalog number 14-447) in 40 μl of a kinase buffer consisting of 50 mM NaCl, 50 mM Tris, pH 7.4, 10 mM MgCl2, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 0.01% Brij-35, and 0.25 mM ATP. For experiments involving 32P, ATP was reduced to 0.1 mM, and 1 μCi of [γ-32P]ATP was added. The reactions were incubated at 30 °C for 1 h and terminated by the addition of SDS sample buffer, followed by boiling.

Phosphopeptide Analysis by Mass Spectrometry—5 μg of GST-POSH-(53–888) was phosphorylated in vitro with either Akt1 or Akt2 as described above. After the reaction GST-POSH was recovered on glutathione-Sepharose beads and washed twice in 100 mM NaCl, 20 mM Tris, pH 8.0, 1 mM CaCl2, 0.1% Thesit, followed by one wash in 100 mM NaCl, 20 mM Tris, pH 8.0, 1 mM CaCl2, 5 mM DTT. Bound proteins were then denatured and reduced by boiling for 5 min in the above buffer containing 1% SDS. Samples were cooled and alkylated by the addition of iodoacetamide to a final concentration of 10 mM for 30 min in the dark at room temperature. Samples were then diluted 5-fold with 100 mM NaCl, 20 mM Tris, pH 8.0, 1 mM CaCl2, 2% Thesit and digested with 0.5 μl of trypsin (sequencing grade, Promega, Madison, WI) overnight at 37 °C. Acetic acid was then added to 0.25 M followed by acetonitrile to 30%, and the tryptic digests were incubated with 10 μl of immobilized Fe3+ resin (PHOS-Select, Sigma) for 1 h at room temperature with rotation. Fe3+ beads were recovered by centrifugation, washed three times with 250 mM acetic acid, 30% acetonitrile, once with H2O, and then eluted with 0.4 M NH4OH. Eluted peptides were analyzed by high resolution MALDI-TOF mass spectrometry performed on a Bruker Ultraflex MALDI-TOF/TOF instrument.

For identification of minor phosphorylation sites, 10 μg of S304A-GST-POSH-(1–888) was phosphorylated in vitro with either Akt1, Akt2, or no added kinase at 30 °C for 2 h, and the reaction products were subjected to SDS-PAGE and Coomassie staining. The bands containing GST-POSH were excised and digested in-gel with trypsin, and phosphopeptides were recovered by binding to a titanium dioxide matrix, as directed by the manufacturer (Phos-trap, PerkinElmer Life Sciences). Eluted peptides were analyzed by MALDI-TOF mass spectrometry, as above.

Rac Binding—35S-Labeled L61Rac was prepared by in vitro transcription and translation using [35S]methionine (PerkinElmer Life Sciences) and the Tnt T7 Quick Coupled Transcription/Translation System as described by the manufacturer (Promega), and used without further purification.

For the phosphorylation time course experiment, kinase reactions were performed in duplicate containing 2 μg of purified GST-POSH-(53–888) protein and 40 ng of active Akt in 20 μl of 50 mM KCl, 10 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM DTT, and 1 mM ATP. The reactions were set up on ice and transferred to 30 °C for varying times (0–60 min), following which the reactions were returned to ice, and 1 unit of apyrase was added to remove residual ATP. Glutathione-Sepharose (30 μl of 50% slurry in phosphate-buffered saline, 1% BSA, 0.1% Thesit) and 35S-labeled L61 Rac (1 μl, diluted 1:10 in phosphate-buffered saline, 0.1% Thesit) were then added, and the reactions were rotated at 4 °C for 2 h to allow protein binding. The samples were then centrifuged, and the Sepharose pellets were washed four times with ice-cold phosphate-buffered saline, 0.1% Thesit, to remove unbound protein. Washed samples were then resuspended in SDS sample buffer, followed by boiling. Aliquots of each sample were analyzed by scintillation counting, SDS-PAGE followed by Coomassie staining and autoradiography, and SDS-PAGE followed by immunoblotting with phospho-Akt substrate antibody (Cell Signaling Technology, Danvers, MA).

Caspase Assays—24 h prior to transfection, MDA-MB-231 cells were plated at 20,000 cells/well in 96-well plates. The next day cells were transfected with pEBG or pEBG-POSH-S304 mutants using Superfect (Qiagen, Valencia, CA) according to manufacturer’s protocol. 24 h post-transfection, cells were lysed and assayed for caspase activity by the addition of 100 μl of Caspase-Glo® 3/7 reagent to each well according to the manufacturer’s protocol (Promega, Madison, WI). Reactions were done in triplicate, including no treatment, transfection vehicle alone, and vector alone controls. The contents of the wells were mixed using a plate shaker for 30 s and incubated at room temperature for 1 h. Luminescence was measured using a luminometer.

RESULTS

Akt Prevents POSH-induced Apoptosis—Overexpression of POSH induces apoptosis in a variety of different cell types, including Swiss 3T3 and NIH 3T3 fibroblasts, neuronally differentiated PC12 cells, primary rat sympathetic neurons, and HEK293 cells (1, 2, 4, 6–9). Here we examined the effects of
Phosphorylation of POSH by Akt

**A.**

![GFP, POSH, POSH + myr Akt](image)

**B.**

![Graph showing survival rates](image)

**C.**

![Graph showing survival rates](image)

**FIGURE 1.** Akt protects cells from POSH-induced apoptosis. A, MDA-MB-231 cells were micro-injected with vectors encoding GFP alone or GFP plus POSH with and without myr-Akt. Still photographs from a time lapse experiment are shown to indicate the morphology of the cells. To quantitate POSH-induced cell death, cells were injected with GFP alone or GFP-POSH with and without myr-Akt1 (B) or myr-Akt2 (C). GFP-positive cells were examined and counted 4 h post-injection and again at 18 h post-injection. Survival is plotted as the percentage of GFP-positive cells remaining alive at 18 h as compared with 4 h post-injection.

POSH overexpression in an additional cell type, MDA-MB-231 human breast cancer cells. These cells were chosen because they have low levels of endogenous activated Akt,\(^4\) and we wished to examine POSH-induced death in the absence of activated Akt. Preliminary experiments indicated that transfection of POSH or a GFP-POSH fusion construct induced rapid cell death in MDA-MB-231 cells (not shown). In Fig. 1B we quantitate this effect by micro-injecting cells with an expression vector encoding either GFP or GFP-POSH and monitoring subsequent cell survival at 4 and 18 h after injection. Greater than 90% of cells injected with GFP-POSH undergo cell death by 18 h. As seen in other cell types, POSH-induced cell death in MDA-MB-231 cells appears to occur by apoptosis, as evidenced by cell rounding, increased refractivity, nuclear condensation, surface blebbing, and detachment from the plate (see Fig. 1A), whereas control cells injected with GFP alone do not show such morphological changes.

However, when these cells are injected with both GFP-POSH and activated forms of either Akt-1 or Akt-2, apoptosis is dramatically reduced. These constitutively active Akt forms contain a myristoylation sequence (abbreviated as myr-Akt), which directs them to the plasma membrane and results in their activation. Similar results demonstrating rescue of POSH overexpressing neuronal PC12 cells by co-expression of myr-Akt-1 have also been reported (2).

**Akt Directly Phosphorylates POSH**—Akt interacts with several members of the PI3K complex to exert a variety of anti-apoptotic effects. We wanted to ask whether Akt might directly phosphorylate POSH. We examine this question in Fig. 2. In Fig. 2A HEK293T cells were transfected with pEBG-POSH, encoding a GST-POSH fusion protein, plus and minus an expression vector for myr-Akt1. Cells were treated with the caspase inhibitor benzoyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (Fluor) to prevent apoptosis. After 24 h the cells were lysed, and GST-POSH was recovered on glutathione-agarose. Analysis of recovered protein by Western blotting with antibodies to GST shows similar levels of GST-POSH protein expression with and without Akt (Fig. 2A, upper panel, compare lanes 2 and 3). To examine possible POSH phosphorylation, we made use of a commercially available phospho-specific antibody that recognizes a variety of Akt substrates in their phosphorylated forms (PAS; Cell Signaling Technology). This PAS antibody shows strong reactivity with GST-POSH precipitated from cells co-expressing myr-Akt1, as compared with cells expressing GST-POSH alone (Fig. 2A, lower panel, compare lanes 2 and 3), indicating that expression of active Akt1 results in POSH phosphorylation in vivo.

To confirm that POSH is directly phosphorylated by Akt1 (versus other kinases activated by Akt1), we attempted to reconstitute Akt phosphorylation of POSH in vitro, using purified proteins. A full-length GST-POSH fusion protein was produced in E. coli, purified, and incubated with purified preparations of active Akt1 or Akt2 in the presence of \(\gamma\)-radiolabeled ATP. Reaction products were then analyzed by SDS-PAGE followed by autoradiography. Fig. 2B shows the results of this experiment. It can be seen that full-length GST-POSH is indeed directly phosphorylated by both Akt1 and Akt2 in vitro, whereas GST alone is unmodified (Fig. 2B, compare lanes 5 and 6 with 2 and 3).

**Akt Phosphorylates POSH within the Rac-binding Domain**—To investigate how phosphorylation might affect POSH function, we sought to identify the precise site(s) of POSH phosphorylation by Akt. Toward this end, we made use of a slightly truncated form of POSH lacking the RING domain (residues 1–52), because previous experiments had indicated that this form must be more efficiently phosphorylated in vitro (not shown).

GST-POSH-(53–888) was phosphorylated by either Akt1 or Akt2 and digested with trypsin, and phosphopeptides were recovered by immobilized Fe\(^{3+}\) affinity chromatography. Eluted peptides were then analyzed by high resolution MALDI-TOF mass spectrometry, with the results presented in Fig. 3A. In both the Akt1- and the Akt2-phosphorylated sam-

\(^4\) S. M. Anderson and M. M. Richert, unpublished data.
Akt Phosphorylates POSH at Multiple Sites—To determine whether Akt may phosphorylate POSH at other sites in addition to Ser-304, we constructed mutant forms of GST-POSH in which Ser-304 was mutated to either alanine, S304A, or aspartate, S304D. These mutant GST-POSH proteins were then tested as Akt substrates in vitro. It can be seen in Fig. 4A that phosphorylation of the Ser-304 mutants is substantially reduced compared with wild type, confirming the mass spectrometry identification of this residue as the major phosphorylation site. S304A and S304D mutants of GST-POSH were still able to incorporate reduced levels of $^{32}$P, however, indicating that although Ser-304 constitutes the major phosphorylation site for Akt, additional minor phosphorylation sites are also present.

To begin to map these additional sites, we constructed a series of smaller GST-POSH proteins containing either N-terminal or C-terminal halves of the protein, or isolated POSH domains, and we tested these as Akt substrates. Fig. 4C shows the results of this analysis. GST proteins containing the N-terminal half of POSH, as well as the isolated Rac-binding domain are efficiently phosphorylated, as is consistent with the mass spectrometry studies described above (Fig. 4C, lanes 3 and 9). None of the four isolated SH3 domains are found to be phosphorylated under these conditions; however, an additional significantly phosphorylated site (or sites) appears to be present within the C-terminal half of POSH (Fig. 4C, lane 4). Note that in the experiment shown, GST-N-POSH was inadvertently under-loaded compared with the other proteins, and thus the phosphorylation of N-POSH appears (misleadingly) low (Fig. 4, C and D, lane 3). In Fig. 4E, phosphorylation of N-POSH and C-POSH is directly compared with more equal protein loadings, and it can be seen that N-POSH is actually phosphorylated to a much greater extent than C-POSH (Fig. 4E, compare lanes 1 and 2). Quantitation of these data indicate that N-POSH incorporated 4.17 times as much $^{32}$P as C-POSH under these conditions, and this may in fact be an underestimate, because Coomassie staining reveals a slightly higher level of C-POSH protein. In addition to the C-terminal half of POSH, a linker region between the RING and the first SH3 domain, residues 53–134, also incorporates $^{32}$P phosphate, although somewhat weakly (Fig. 4C, lane 10).

To identify more precisely the identity of these minor phosphorylation sites, we again turned to mass spectrometry. Full-length GST-POSH S304A protein was phosphorylated in vitro with either Akt1 or Akt2 or no added kinase. Following the reaction, GST-POSH was re-isolated by SDS-PAGE, digested with trypsin, and phosphopeptides purified by binding to a tita-

FIGURE 2. Akt phosphorylates POSH. A, in vivo, HEK-293T cells were transfected with pEBG vector alone or vector encoding GST-POSH (pEBG-POSH) plus and minus a vector encoding constitutively active Akt1 (pCDNA3.1-myr-Akt1), with benzoyloxycarbonyl-VAD present in the culture media to prevent apoptosis. 24 h after transfection cell lysates were obtained and incubated with glutathione-Sepharose to recover GST-POSH, and bound protein was analyzed by immunoblotting with antibodies to GST (lower panel) or PAS antibody (upper panel). B, in vitro, GST and GST-POSH proteins were purified from E. coli and incubated with $\gamma$-[$^{32}$P]ATP and purified preparations of active Akt1 or Akt2 as indicated, followed by SDS-PAGE and autoradiography.
Phosphorylation of POSH by Akt

A.

![Graph showing Akt phosphorylation](image)

B.

![Graph showing ion intensity](image)

C.

| N-Term | Ion | a   | b   | b-Pl | y   | y-Pl | C-Term. | Ion |
|--------|-----|-----|-----|------|-----|------|---------|-----|
| 1      | K   | 101.1 | 129.1 | 147.1 | 13  | K    |         |     |
| 2      | R   | 257.2 | 285.2 | 281.2 | 12  | N    |         |     |
| 3      | H   | 394.3 | 422.3 | 332.2 | 11  | A    |         |     |
| 4      | S   | 561.3 | 589.3 | 491.3 | 463.2 | 10  | M    |     |
| 5      | F   | 708.3 | 736.3 | 638.4 | 564.3 | 9   | T    |     |
| 6      | T   | 809.4 | 837.4 | 739.4 | 677.4 | 8   | L    |     |
| 7      | S   | 924.4 | 826.4 | 764.4 | 7   | S    |         |     |
| 8      | L   | 1037.5 | 938.5 | 865.4 | 6   | T    |         |     |
| 9      | T   | 1138.5 | 1040.5 | 1012.5 | 5   | F    |         |     |
| 10     | M   | 1171.6 | 1179.5 | 1081.5 | 4   | S*   |         |     |
| 11     | A   | 1242.6 | 1316.6 | 1218.6 | 3   | H    |         |     |
| 12     | N   | 1454.7 | 1356.7 | 1472.7 | 1374.7 | 2   | R    |     |
| 13     | K   | 1484.8 | 1600.8 | 1502.8 | 1   | K    |         |     |
Phosphorylation of POSH by Akt

Despite 733–768 of POSH, containing a single phosphate group (m/z predicted 3552.77, observed 3552.77). MS-MS analysis of this peak was unsuccessful; however, Scansite predicts Ser-734 as a possible Akt phosphorylation site. Peaks B and D do not appear to be derived from POSH and were not analyzed further.

Residue Ser-125 lies in the linker domain between the RING domain and the first SH3 domain of POSH, and likely explains the weak phosphorylation of this domain seen in 4C (lane 10). The other two phosphopeptides containing Ser-734 and Ser-799, are located in the C-terminal half of POSH, between the third and fourth SH3 domains. Although no function has yet been linked to this region of POSH, this result can explain the phosphorylation of GST-C-POSH seen in 4, C and E.

Akt Phosphorylation of POSH Reduces Rac Binding—We have identified serine 304 in POSH as a major phosphorylation site for both Akt1 and Akt2. Because this residue lies within the Rac-binding domain of POSH, we wished to determine how phosphorylation might affect Rac binding. This question is addressed in Fig. 6. In this experiment, GST-POSH-(53–888) was used as a substrate for in vitro phosphorylation by Akt2. Phosphorylation reactions were incubated at 30 °C for different lengths of time (5–60 min, as indicated) or remained on ice (indicated as time 0 in Fig. 6). In addition, control reactions lacking either ATP or Akt2 were employed. Following the incubation period, apyrase was added to remove residual ATP, followed by addition of 35S-labeled L61–Rac. Glutathione-agarose was then added to recover GST-POSH and any 35S-Rac bound to it. The samples were washed, and bound proteins were analyzed by SDS-PAGE followed by Coomassie staining and autoradiography. In addition, aliquots of each sample were analyzed by scintillation counting to quantify 35S-Rac binding and by immunoblotting with PAS antibody to reveal POSH phosphorylation. It can be seen that as phosphorylation of POSH increases, the ability to bind 35S-Rac decreases (Fig. 6, lanes 5–12). We note also that Rac binding is reduced even in the samples incubated with Akt at 0 °C (Fig. 6, time 0, lanes 5 and 6). We have observed that some phosphorylation of POSH by Akt occurs at 0 °C under these conditions (data not shown). Furthermore, it appears that the control sam-

FIGURE 4. Akt phosphorylates POSH at multiple sites. A, wild type GST-POSH or GST-POSH fusion proteins containing point mutations in serine 304 to either alanine (S304A) or aspartate (S304D) were incubated with activated Akt1 or Akt2 in the presence of [γ-32P]ATP and then analyzed by SDS-PAGE and autoradiography. Although Ser-304 mutations substantially reduce phosphorylation, they do not completely eliminate it. B, domain structure of POSH is diagrammed, and the regions of POSH present in a series of GST-POSH fusion proteins are indicated. RBD, Rac-binding domain. These fusion proteins are used as substrate in C, C–E, GST-POSH fusion proteins containing either full-length POSH (lane 2) or smaller regions of POSH (lanes 3–10) were incubated with activated Akt in the presence of [γ-32P]ATP. GST fusion proteins were then recovered by binding to glutathione-agarose and analyzed by SDS-PAGE, followed by Coomassie staining (D) and autoradiography (C). Trace amounts of BSA adsorbed nonspecifically to the glutathione-agarose beads are also evident in the Coomassie panel. Note that GST-N-POSH was inadvertently underloaded in C and D (lanes 3), so it has been assayed again in E to allow direct comparison of GST-N-POSH with GST-C-POSH.

FIGURE 3. Akt phosphorylates POSH within the Rac-binding domain. GST-POSH-(53–888) was phosphorylated in vitro by either Akt1 or Akt2 and then digested with trypsin. Phosphopeptides were purified by affinity chromatography with immobilized Fe3+ resin and analyzed by MALDI-TOF mass spectrometry. A, MALDI-TOF spectra are shown of peptides recovered from GST-POSH phosphorylated with Akt1 or Akt2, as indicated. Peaks with m/z values labeled match values calculated for phosphopeptides derived from KRHSFTSLTMANK, corresponding to residues 301–313 of POSH. B, peak of m/z 1600.8 from the Akt2 sample in A was further analyzed by tandem MS-MS. A TOF-TOF spectrum is shown, with higher sensitivity shown in the inset. Labeled peaks match ions expected for phosphorylation at serine 304 (KRHSFTSLTMANK). C, table matching the peaks identified in B with the predicted ion series, as indicated.
Phosphorylation of POSH by Akt

**FIGURE 5. Identification of minor phosphorylation sites in POSH.** Full-length GST-POSH-(1–888) containing an S304A point mutation was phosphorylated in vitro by either Akt1, Akt2, or no added kinase, and the reaction products were separated by SDS-PAGE. GST-POSH protein bands were excised and digested with trypsin, and phosphopeptides were purified by affinity chromatography with a titanium dioxide matrix. Eluted peptides were analyzed by MALDI-TOF mass spectrometry. Prominent peaks observed in the sample treated with Akt2 that are absent in the control are labeled as peaks A–E. These peaks are also present in the Akt1 sample, although at lower intensity, and are candidates for phosphopeptides. Tandem MS-MS analysis was successfully performed for peaks A and C and confirmed their identities as phosphopeptides (not shown). Peak A corresponds to the peptide VQSWSPPPVR, residues 123–131 of POSH, containing a single phosphate group (m/z predicted 1135.53, observed 1135.52). Peak C corresponds to the peptide KASSLSAVPIAPPR, residues 797–812 of POSH, containing a single phosphate group (m/z predicted 1685.86, observed 1685.86). Peak E appears to correspond to the peptide VSPPASPTLEVELGSAELPLQGAVGPELPPGGGHGR, residues 733–768 of POSH, containing a single phosphate group (m/z predicted 3552.77, observed 3552.77), however MS-MS was unsuccessful for this peak. Peaks B and D do not appear to have originated from POSH, and they were not analyzed further.

Phosphoryomic Mutation of POSH Ser-304 Reduces Rac Binding and Apoptosis—To further confirm that serine 304 of POSH is the major phosphorylation site used by Akt, and to investigate the functional consequences of phosphorylation by Akt at this site, we further studied the behavior of POSH containing Ser-304 mutations. In Fig. 7A, we compare the ability to bind $^{35}$S-L61-Rac of wild type POSH with mutant versions containing either alanine (S304A), aspartate (S304D), or glutamate (S304E). We find that mutation of Ser-304 to alanine does not appear to disrupt Rac binding (Fig. 7A, compare lanes 5 and 6 with lanes 7 and 8). In contrast, mutation to either aspartate or glutamate, which contain fixed negative charge and may therefore mimic phosphorylation at this site, significantly reduces Rac binding to POSH (compare lanes 9–12 with 5–8). Similar results are observed in vivo with endogenous Rac. In Fig. 7B, expression vectors for GST-POSH containing either the S304A or S304D mutations were transfected into HEK 293T cells, and 24 h later GST-POSH and any proteins bound to it were recovered on glutathione-agarose. Immunoblotting of bound proteins shows that the POSH S304A mutant retains the ability to bind Rac, whereas Rac binding to the S304D mutant, expressed at similar levels, is drastically reduced (Fig. 7B, compare lanes 2 and 3). The functional consequences of reduced Rac binding are evident in Fig. 7C. Here MDA-MB-231 human breast cancer cells were transfected with expression vectors for Ser-304 POSH mutants or empty vector, and caspase activity was measured 24 h later as an indication of apoptosis. It can be seen that the POSH S304A mutant retains the ability to induce apoptosis, as measured by caspase activation, whereas the POSH S304D mutant showed only background levels of caspase activity for this assay (Fig. 7C, compare lanes 1–3). Taken together, our findings indicate that phosphorylation of POSH at serine 304 by Akt strongly reduces the ability of POSH to bind Rac and thereby reduces the ability of POSH to promote apoptosis.

**DISCUSSION**

POSH participates in apoptotic signaling by binding activated Rac and linking it to a kinase cascade pathway that results in JNK activation. POSH acts as a scaffold protein in a multi-protein signaling complex that has recently been termed “PJAC” (for POSH-JIP apoptotic complex). In this signal transduction pathway POSH directly binds both active Rac and one of the mixed lineage kinases (MLK1, -2, -3, or DLK), and the subsequent interaction of active Rac with MLK induces auto-phosphorylation and activation of MLK. POSH also directly binds a second scaffold protein JIP (JNK-interacting protein 1 or 2), and JIP completes the complex by binding MKK4 or 7 and JNK (1, 2, or 3). Activated MLK can then phosphorylate and activate MKK4/7, and active MKK4/7 in turn phosphorylates and activates JNK. JNKs activated through this pathway go on to trigger apoptosis through release of cytochrome c from mitochondria and the subsequent activation of caspases.

Apoptosis is highly regulated through multiple mechanisms and at multiple sites. The pro-survival kinase Akt has emerged...
We identify Ser-125, Ser-799, and possibly Ser-734 as minor sites of phosphorylation by Akt, other minor sites are also present.

POSH strongly reduces POSH phosphorylation by Akt but does not completely eliminate it. Although serine 304 is a major site of phosphorylation by Akt, other minor sites are also present. We identify Ser-125, Ser-799, and possibly Ser-734 as minor phosphorylation sites. We further show that phosphorylation of POSH by Akt leads to loss of its ability to bind active Rac, and would thereby be predicted to block transmission of the apoptotic signal from Rac to the kinase cascade that results in activation of JNK. Support for this prediction is obtained by observing the behavior of POSH Ser-304 mutants. POSH S304A retains the ability to bind Rac, whereas S304D and S304E mutants show reduced Rac binding, indicating these mutants may mimic the effects of phosphorylation at that site. Although S304A POSH is still able to induce apoptosis as measured by caspase activation, the POSH S304D mutant did not show any increase in apoptosis above background levels in the assay.

Akt appears to regulate the PJAC apoptotic pathway at multiple sites and by multiple mechanisms, as summarized diagrammatically in Fig. 8. We report here that phosphorylation of POSH by Akt at serine 304 reduces the ability of POSH to bind to Rac. Previous studies by other authors have reported Akt phosphorylation of other PJAC components. Kwon et al. (30) have described Akt phosphorylation of Rac1 at serine 71, which prevents Rac from being activated by blocking its ability to bind GTP. Akt has also been reported to phosphorylate MLK3 on serine 674 and MKK4 on serine 78, both of which result in decreased kinase activity and subsequent reduced JNK activation (31, 32). In addition to modulation of PJAC components by phosphorylation, it appears that Akt may also regulate PJAC by nonenzymatic mechanisms. As discussed earlier, Akt2 (but not Akt1) can directly bind to POSH, and Akt2 binding appears to block the binding of MLK3, inhibiting the apoptotic pathway by preventing PJAC assembly (23). Finally, in another isoform-specific nonenzymatic mechanism, Akt1 (but not Akt2) can bind directly to JIP1, an interaction that has been reported to reduce JNK activation and apoptotic signaling (33).

In our experiments examining the ability of Akt-phosphorylated POSH to bind Rac, it is possible that some phosphorylation of Rac might also have occurred. We believe this is unlikely because apyrase was added to deplete ATP prior to the addition of 35S-Rac. Furthermore, Akt-mediated phosphorylation of Rac is unlikely to contribute to the results we have obtained here because we have employed a constitutively active form of Rac that already contains bound GTP and lacks the ability to hydrolyze it (Rac Q61L).

The multiple regulatory actions of Akt upon PJAC suggest that in order for JNK to become activated through the PJAC pathway and trigger apoptosis, Akt activity must be low, or these multiple levels of regulation by Akt must be overcome. A degree of cross-talk between the JNK and Akt pathways appears to exist. There is evidence that JNK can directly oppose actions of Akt, and furthermore that under some conditions JNK activation may lead to a reduction in the levels of activated Akt. For example, Akt phosphorylation of Bad and FOXO3a provides a binding site for 14-3-3 proteins and results in sequestration of these pro-apoptotic proteins in the cytoplasm, where they are unable to act. JNK can phosphorylate 14-3-3 and cause release of bound Bad and FOXO3a, antagonizing the effect of Akt (34). In another apparent example of cross-talk, Sunters et al. (35) have reported that JNK activation following treatment with paclitaxel leads to reduced levels of active Akt. Other examples of cross-talk between these pathways likely await discovery.

Our findings here identify POSH phosphorylation as a novel

**Phosphorylation of POSH by Akt**

![Graph showing Akt phosphorylation of POSH reduces its ability to bind Rac.](image-url)
**Phosphorylation of POSH by Akt**

**FIGURE 7.** Phosphomimetic mutation of POSH Ser-304 reduces Rac binding and apoptosis. A, in vitro binding of Rac to GST-POSH or Ser-304 mutants of GST-POSH. Duplicate samples containing equal amounts of purified GST (lanes 3 and 4) or full-length GST-POSH (lanes 5–12) were incubated with 35S-L61-Rac and glutathione-agarose for 2 h at 4 °C. Agarose beads were then washed to remove unbound proteins, and bound proteins were analyzed by SDS-PAGE followed by Coomassie staining and autoradiography. The top panel shows quantitation of 35S-Rac binding by scintillation counting of aliquots of each sample. The 2nd panel reveals 35S-Rac binding by autoradiography, and the Coomassie stain of the same gel is shown at the bottom. Lane numbers are indicated at the bottom of the panel. Lane 1 contains 20% of the 35S-L61-Rac used in each of the binding reactions; lane 2 contains molecular weight markers; lanes 3 and 4 contain GST as a control for specificity of Rac binding; lanes 5 and 6 contain wild type GST-POSH; lanes 7 and 8 contain S304A mutant GST-POSH; lanes 9 and 10 contain S304D mutant GST-POSH; and lanes 11 and 12 contain S304E mutant GST-POSH. B, in vivo binding of Rac to GST-POSH. HEK293T cells were transfected with expression vectors for S304A-GST-POSH (lane 2), S304D-GST-POSH (lane 3), or empty vector (lane 1), and 24 h later cells were lysed, and GST-POSH and any proteins bound to it were recovered by binding to glutathione-agarose. Agarose beads were then washed to remove nonspecifically bound proteins, and the remaining bound proteins analyzed by SDS-PAGE followed by immunoblotting and chemiluminescent visualization. The upper panel shows reactivity with anti-POSH antibodies, and the lower panel shows reactivity to anti-Rac antibodies. C, induction of apoptosis by POSH Ser-304 mutants. MDA-MB-231 cells were transfected with expression vectors for S304A-GST-POSH (lane 2), S304D-GST-POSH (lane 3), or empty vector (lane 1). 24 h later cells were lysed and assayed in triplicate for activity of effector caspases 3 and 7 as an indication of apoptosis using a luminescent assay as described under “Materials and Methods.” In the lower panel, parallel samples were analyzed by immunoblotting with antibodies to POSH, to ensure equivalent expression.

**FIGURE 8.** Regulation of PJAC by Akt. Shown are models indicating both enzymatic (A) and nonenzymatic (B) regulation of PJAC by Akt. A, Akt regulates PJAC through phosphorylation at multiple sites. We report here that Akt phosphorylates POSH on Ser-304 (shown by large arrow) and other minor sites (shown by smaller arrows) to result in a loss of its ability to bind Rac. Others have reported that Akt also phosphorylates Rac, MLK3, and MKK4 (additional small arrows), in each case resulting in decreased JNK activation and reduced apoptosis. B, Akt apparently also regulates PJAC through nonenzymatic binding interactions. Akt2 specifically binds POSH and prevents MLK3 binding, and Akt1 specifically binds JIP1. Both interactions lead to reduced JNK activation. See “Discussion” for more details.
mechanism for regulation of apoptosis by the pro-survival kinase Akt. The extensive regulation of the PJAC apoptotic pathway by Akt at multiple sites and through multiple mechanisms suggests that the interplay between Akt and the PJAC pathway is of significant physiological importance.

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