Hsp27 Regulates Akt Activation and Polymorphonuclear Leukocyte Apoptosis by Scaffolding MK2 to Akt Signal Complex*

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Rui Wu‡1, Hina Kausar‡1, Paul Johnson‡, Diego E. Montoya-Durango‡, Michael Merchant‡, and Madhavi J. Rane‡§2

From the Departments of ‡Medicine and §Biochemistry and Molecular Biology, University of Louisville, Louisville, Kentucky 40202

We have shown previously that Akt exists in a signal complex with p38 MAPK, MAPK-activated protein kinase-2 (MK2), and heat shock protein 27 (Hsp27) and MK2 phosphor-ylates Akt on Ser-473. Additionally, dissociation of Hsp27 from Akt, prior to Akt activation, induced polymorphonuclear leukocyte (PMN) apoptosis. However, the role of Hsp27 in regulating Akt activation was not examined. This study tested the hypothesis that Hsp27 regulates Akt activation and promotes cell survival by scaffolding MK2 to the Akt signal complex. Here we show that loss of Akt/Hsp27 interaction by anti-Hsp27 antibody treatment resulted in loss of Akt/MK2 interaction, loss of Akt-Ser-473 phosphorylation, and induced PMN apoptosis. Transfection of myristoylated Akt (AktCA) in HK-11 cells induced Akt-Ser-473 phosphorylation, activation, and Hsp27-Ser-82 phosphorylation. Co-transfection of AktCA with Hsp27 short interfering RNA, but not scrambled short interfering RNA, silenced Hsp27 expression, without altering Akt expression in HK-11 cells. Silencing Hsp27 expression inhibited Akt/MK2 interaction, inhibited Akt phosphorylation and Akt activation, and induced HK-11 cell death. Deletion mutagenesis studies identified acidic linker region (amino acids 117–128) on Akt as an Hsp27 binding region. Deletion of amino acids 117–128 on Akt resulted in loss of its interaction with Hsp27 and MK2 but not with Hsp90 as demonstrated by immunoprecipitation and glutathione S-transferase pulldown studies. Co-transfection studies demonstrated that constitutively active MK2 (MK2EE) phosphorylated Aktwt (wild type) on Ser-473 but failed to phosphorylate AktΔ117–128 mutant in transfixed cells. These studies collectively define a novel role of Hsp27 in regulating Akt activation and cellular apoptosis by mediating interaction between Akt and its upstream activator MK2.

Apoptosis or programmed cell death is a series of events in a cell that leads to its death. Human polymorphonuclear leukocytes (PMN)3 take part in host defense mechanisms against infection and inflammatory diseases. Inappropriate termination of PMN activation or failure to remove apoptotic PMNs results in inflammation. This apoptotic process has been suggested to represent an in vivo mechanism limiting oxidant-induced tissue injury caused by PMNs at the sites of inflammation. Although PMNs are constitutively committed to apoptosis from the time they enter circulation, the rate of apoptosis is not fixed. We reported that interleukin-8, granulocyte-macrophage colony-stimulating factor, LTB4, and bacterial lipopolysaccharide (LPS) delay constitutive PMN apoptosis through the activation of the serine/threonine kinase Akt (1, 2). We demonstrated that p38 mitogen-activated protein kinase (MAPK) activity is required for Akt phosphorylation and activation (3). Additionally, we showed that Akt exists in a signaling module with p38 MAPK, MAPK-activated protein kinase-2 (MK2), and heat shock protein 27 (Hsp27) (3).

Heat shock proteins represent a group of chaperone proteins that protect the cells against a variety of stresses. Besides being involved in functioning as a chaperone, Hsp27 has also been shown to regulate stability of the cytoskeleton, cell motility (4–7), and apoptosis (8–13). When overexpressed in tumor cells, Hsp27 increases their tumorigenicity by overexpressing MMP-9 expression and down-regulating Src tyrosine kinase Yes expression (14–16) and protects against apoptotic cell death triggered by various stimuli, including cytotoxic drugs and ligation of the Fas/Apo-1/CD95 death receptor (17–19). Mice overexpressing Hsp27 were protected from lethal ischemia/reperfusion injury compared with their negative litter-mates (20). Possible mechanisms of Hsp27 anti-apoptotic activity are proposed to result from its activity as a molecular chaperone. Hsp27 binds to and inactivates the pro-apoptotic molecules Smac, caspase 3, caspase 9, and cytochrome c (21–25). Hsp27-mediated suppression of Bid translocation to the mitochondria correlates with an inhibition of cytochrome c release (25). Hsp27 has also been shown to promote survival

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: University of Louisville, 570 S. Preston St., Baxter I Bldg. South, 102C, Louisville, KY 40202. Tel.: 502-852-0014; Fax: 502-852-4384; E-mail: mjran01@gwise.louisville.edu.

§ The abbreviations used are: PMN, polymorphonuclear leukocyte; Hsp27, heat shock protein 27; PKD1, phosphoinositide-dependent kinase-1; PKD2, phosphoinositide-dependent kinase-2; MAPK, mitogen-activated protein kinase; MK2, MAPK-activated protein kinase-2; HK-11 cells, human renal proximal tubular cells; HEK-293, human embryonic kidney cells; AktCA, c-Myc-tagged myristoylated constitutively active Akt; IEF, isoelectric focusing; MES, 4-morpholineethanesulfonic acid; PH, pleckstrin homology; PMSF, phenylmethlysulfonyl fluoride; fMLP, formylmethionylleucylphe

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pathways by modulating IKK complex stability and activity. Parcellier et al. (26) demonstrated that Hsp27 mediates NF-KB activation and cell survival by promoting the proteasomal degradation of polyubiquitinated IKK. Phosphorylated Hsp27 has been shown to bind an adaptor protein Daxx and to inhibit Fas-mediated apoptosis (27). Additionally, phosphorylation of Hsp27 has been shown to be required for proper maintenance of cell adhesion and inhibition of renal epithelial cell apoptosis (28). Furthermore, Sheth et al. (13) showed that introduction of recombinant Hsp27 caused delay of PMN apoptosis; however, mechanisms regulating this delay of PMN apoptosis were not determined.

We recently demonstrated direct protein/protein interaction between Akt/Hsp27 (3, 8). The physical association of Hsp27 with Akt is a critical determinant of PMN survival, as removal of Hsp27 from the Akt signal module prevented Akt phosphorylation and activation and resulted in accelerated PMN apoptosis suggesting an important role for Hsp27 in regulating Akt activity (8). MK2 has been shown to bind and phosphorylate Hsp27 (29), and MK2 is PDK2 for Akt in human PMNs (3). Recently, Zheng et al. (30) demonstrated that MK2 is required for p38 MAPK and Hsp27 interaction; however, association of Hsp27 and Akt was not dependent on MK2. Hence we hypothesized that Hsp27 regulates Akt activation and apoptosis by scaffolding MK2 to the Akt signal complex.

Akt contains an N-terminal pleckstrin homology (PH) domain and a catalytic kinase domain (residues 1–116 and 148–411 respectively) linked by a highly acidic linker region (residues 117–147). The C-terminal tail region lies between residues 412 and 480. Phosphoinositides are known to bind the PH domain of Akt and recruit it to the plasma membrane for full activation by PDK1 and PDK2 (28, 31–33). In the present study we show that amino acids 117–128 within the acidic linker region (mutating the KpnI site) was 5’-AGGCAGGAAGAAGAGTCAGGGGCTGAGAG-3’, and the selection primer for pUseAktwt (mutating the KpnI site) was 5’-GTTAAAGCTTGAATCCGA-GCTCG-3’. Cloning and mutation were confirmed by DNA sequencing.

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Isolation of PMNs and Culture Conditions—PMNs were isolated from venous blood obtained from healthy volunteers as described previously (3, 8). PMN preparations routinely contained >95% PMNs, as determined by morphology, and were >99% viable by trypan blue dye exclusion. PMNs were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1-glutamine, penicillin, and streptomycin and incubated for the indicated times at 37 °C in 5% CO2.

Generation of pUseAktwt/AktΔ117–128 Mutant by Site-directed Mutagenesis—In-frame deletion of amino acids 117–128 from pUseAktwt cDNA was carried out using the Transformer site-directed mutagenesis kit from BD Biosciences according to the manufacturer’s instructions. The in-frame deletion primer was 5’-AGGCAGGAAGAAGAGTCAGGGGCTGAGAG-3’, and the selection primer for pUseAktwt (mutating the KpnI site) was 5’-GTTAAAGCTTGAATCCGA-GCTCG-3’. Cloning and mutation were confirmed by DNA sequencing.

Subcloning and Purification of GST Beads and Recombinant Proteins—AktΔ117–128 was excised from pUSEAktΔ117–128 with restriction enzymes BamHI/Pmel and ligated into BamHI/Smal sites of pGEX-4T-2 (GE Healthcare) vector. Generation of GST-Hsp27pGEX-5X-2, GST-AktpGEX-4T-2, was described previously (8). GST-pGEX-4T-2, GST-Hsp27pGEX-5X-2, GST-Akt-pGEX-4T-2, and GST-AktΔ117–128pGEX-4T-2 cDNAs were transformed into Escherichia coli BL21(DE3)PlysS, and the expression and purification of GST, GST-Hsp27, GST-Aktwt, and GST-AktΔ117–128 fusion proteins were performed as described previously (8). pRSETA vector was digested with restriction enzyme EcoRI followed by generation of a blunt end by treatment with Klenow enzyme followed by digestion with BamHI restriction enzyme. This vector was then ligated to either Aktwt or AktΔ117–128 which were excised from pUSEAktwt or pUSEAktΔ117–128 with restriction enzymes BamHI/Pmel. All positive clones were confirmed by DNA sequencing. Expression of pRSET-Aktwt and pRSET-AktΔ117–128 plasmids was carried out in BL21(DE3)pLysS chemically competent E. coli cells, and protein was purified using the ProBond purification system (Invitrogen).

Immunoblot Analysis—Immunoblotting procedures were performed as described previously (3, 8). Fifty μg of protein was subjected to 10% SDS-PAGE and immunoblot analysis with anti-pAktSer-473 (1:1000, Santa Cruz Biotechnology), anti-Akt (1:1000, Santa Cruz Biotechnology), anti-Hsp27 (StressGen), anti-Ser(P)-82Hsp27 (Cell Signaling), anti-c-Myc (Cell Signaling), anti-MK2 (Sigma), and anti-Hsp90 (Santa Cruz Biotechnology) antisera (3, 8).

Gel Filtration Chromatography—The system used for the size exclusion chromatography experiments consisted of a
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HiPrep 26/60 Sephacryl S-300 HR packed chromatography column connected to an AKTA purifier 10 liquid chromatography system (Amersham Biosciences), equipped with a Frac-900 automated fraction collector, and controlled by the UNICORN version 4.00 software (Amersham Biosciences). Prior to chromatography, the column was equilibrated in 50 mM Tris-CI, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1.5 mM MgCl2, 5% glycerol, 0.5% Triton X-100. For the chromatographic separation of the samples, control and fMLP (0.3 μM)-stimulated PMNs were harvested and resuspended in AKT lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 20 mM sodium orthovanadate, 10 μM p-nitrophenol phosphate, 20 mM NaF, 5 mM PMSF, 21 μg/ml aprotinin, and 5 μg/ml leupeptin. The cell lysate was cleared by centrifugation at 14,000 rpm for 15 min. Next, 3–5 ml of total cleared cell lysate was injected onto the column with a manual injection through a 50-ml capacity superloop system (Amersham Biosciences). Isocratic elution column with a manual injection through a 50-ml capacity superloop system (Amersham Biosciences). Isocratic elution chromatography, the column was equilibrated in 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1% (v/v) Triton X-100. Appropriate GST beads (10 μl) were added to the lysates and incubated at 4 °C for 1 h with shaking. The beads were washed three times with Krebs buffer, and 15 μl of 2X Laemmli buffer was added to each tube. The samples were boiled for 3 min and then subjected to 10% SDS-PAGE. Proteins were transferred onto nitrocellulose and immunoblotted with appropriate antibodies.

GST Pulldown Assay with Recombinant Proteins—Appropriate GST beads (10 μl) were added to 50 μl of kinase buffer (20 mM HEPES, 10 mM MgCl2, 10 mM MnCl2) containing 50 ng of appropriate recombinant protein. The samples were incubated at 4 °C for 1 h with shaking. The beads were washed three times with Krebs buffer, and 15 μl of 2X Laemmli buffer was added to each tube. The samples were boiled for 3 min and then subjected to 10% SDS-PAGE and immunoblotting. Recombinant GST-Akt-PH domain (1-149 amino acids) was first conjugated with glutathione-Sepharose beads by incubating the two at 4 °C for 1 h with shaking. These beads were then incubated with 50 ng of recombinant Hsp27 as described above.

Cell Culture—HK-11 cells (human renal tubular epithelial cells) immortalized by transduction with adenovirus 12-SV40 were obtained from Dr. Racusen (34). Cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Invitrogen) supplemented with 5% fetal calf serum (Sigma) and penicillin/streptomycin (100 units/ml) (Invitrogen). Fresh growth medium was added to cells every 3–4 days until confluent. HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/ml) (Invitrogen).

cDNA Transfections—HK-11 or HEK-293 cells were plated on 6-well trays a day prior to performing transfections, to achieve 60% confluence. On the day of transfection appropriate cells were washed with serum-free RPMI 1640 medium. One μg of appropriate cDNA was transfected into these cells using GenePORTER reagent according to the manufacturer’s protocol (Gene Therapy Systems). Twenty four hours after transfection cells were lysed in 100 μl of Akt lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 20 mM sodium orthovanadate, 10 μM p-nitrophenol phosphate, 20 mM NaF, 5 mM PMSF, 21 μg/ml aprotinin, and 5 μg/ml leupeptin, and proteins concentrations were determined. Protein lysates (50 μg) were subjected to SDS-PAGE and immunoblot analysis or to immunoprecipitation studies.

Construction of Hsp27 siRNA—Construction of siRNA was performed as described previously (35). Hsp27 siRNA was generated using Silencer® siRNA construction kit (Ambion, Austin, TX). Hsp27 sequence 5’-AACACCAAG-GATGGCGTGTGGT-3’ was targeted to generate Hsp27 siRNA. The sense and antisense siRNA oligonucleotides used to generate Hsp27 siRNA were 5’-AACACCAAG-GATGGCGTGTGGT-3’ (sense) and 5’-AACACCAAG-GATGGCGTGTGGT-3’ (antisense). The Hsp27 siRNA and a scrambled siRNA were transfected by using GenePORTER reagent as outlined above.
Assessment of Cell Viability—Cell viability was measured by assessment of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction (Sigma) as described previously (36, 37). The soluble form of MTT was reduced by mitochondria of live cells, resulting in a water-insoluble salt. Product formation was monitored by reading absorbance at 540 nm using a microplate reader.

Akt Immunoprecipitation Assays—Appropriate cells were lysed in Akt lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 20 mM sodium orthovanadate, 10 μM p-nitrophenol phosphate, 20 mM NaF, 5 mM PMSF, 21 μg/ml aprotonin, and 5 μg/ml leupeptin. Following centrifugation at 15,000 × g for 15 min at 4 °C, cleared lysates were incubated with 20 μl of anti-Akt PH domain agarose beads or with mouse isotype control antibody beads as described previously (8). Immunoprecipitated proteins were eluted with 40 μl of 2× Laemmli dye. Samples were boiled for 2 min; beads were precipitated by a quick spin in a picofuge, and 40 μl supernatant containing eluted proteins was subjected to SDS-PAGE and immunoblot analysis.

Akt Kinase Assay—HK-11 cells were transfected with pUSE vector or pUSEAktCA (c-Myc-tagged myristoylated constitutively active Akt; Upstate Biotechnology, Inc.) or pUSEAktCA along with Hsp27siRNA. Transfected lysates were subjected to anti-Akt immunoprecipitation (as described above). Immunoprecipitated Akt was subjected to an in vitro Akt kinase assay using histone H2B as substrate as described previously (8).

Annexin V binding was performed as described previously (8). Briefly, 1 × 10⁶ PMNs were washed and resuspended in 100 μl of RPMI 1640 medium without fetal calf serum. The cells were prewarmed for 5 min at 37 °C; anti-Hsp27 antibody (10 μg) or isotype control antibody (10 μg) was then added, and the cells were further incubated for 2 h at 37 °C. PMNs were washed once with RPMI 1640 medium without fetal calf serum and centrifuged at 400 × g for 2 min. Cells were resuspended in ice-cold 1× Binding Buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂) at a concentration of 10⁶ cells/0.3 ml. Next 3 μl of annexin conjugate Apoptinex FITC was added to the cell suspension and incubated at room temperature for 15 min in the dark. The cells were immediately examined using Zeiss Axiovert 100 microscope and LSM 510 software.

RESULTS

Enhanced Akt/Hsp27 Interaction during LPS Delay of PMN Apoptosis—Cultured PMNs undergo constitutive apoptosis such that only about 30% are viable after 24 h. However, treatment of PMNs with lipopolysaccharide (LPS) delays constitutive PMN apoptosis, such that about 70% are viable after 24 h (2). We determined whether increased PMN viability at 24 h in the presence of LPS could be attributed to increased Akt/Hsp27 interaction. Freshly isolated PMNs (zero time control) and PMNs in culture for 24 h with or without LPS were lysed. Lysates were subjected to anti-Akt immunoprecipitation and immunoblotted with anti-Hsp27 antibody. Immunoprecipitated Akt was also immunoblotted with anti-Akt to determine equal immunoprecipitation in every condition. Additionally, as control, zero time PMN lysates were subjected to isotype control antibody immunoprecipitation and immunoblotted with anti-Hsp27 and anti-Akt antisera (Fig. 1, 1st lane). As expected, no Akt or Hsp27 binding was detected in PMN lysates immunoprecipitated with isotype control antibody (Fig. 1, 4th lane). Akt/Hsp27 interaction was detected in zero time PMN lysates subjected to anti-Akt immunoprecipitation (Fig. 1, 3rd lane). Akt-Hsp27 association was markedly reduced in PMNs cultured for 24 h in the absence of LPS (Fig. 1, 2nd lane); however, this association was maintained in PMNs cultured for 24 h in the presence of LPS (Fig. 1, 1st lane). These results suggest there is a need to determine the cause and effect relationship between Akt/Hsp27 interaction and PMN viability, suggesting the importance of Akt/Hsp27 interaction in the regulation of PMN apoptosis during inflammation.

Separation of Akt Signal Components in Control and fMLP-stimulated PMNs—We have previously shown in PMNs that Akt exists in a complex with Hsp27, MK2, and p38 MAPK and that p38 MAPK-dependent kinase MK2 regulates Akt activation by phosphorylating Ser-473 on Akt (8). In addition, we demonstrated that upon fMLP stimulation, Hsp27 dissociates from the Akt signal complex, whereas MK2 and p38 MAPK continue to associate with Akt (3, 8). To estimate percentage of Hsp27 or MK2 or p38 MAPK from PMNs that associate with Akt, we subjected control and fMLP-stimulated PMN lysates to gel filtration chromatography. Fractions collected were immunoblotted with anti-Hsp27, anti-MK2, anti-p38 MAPK, and anti-Akt (Fig. 2A). The results demonstrated that Hsp27 eluted in the same fractions as Akt in control PMN lysates. In addition, MK2 and p38 MAPK eluted in the same fractions as Hsp27 and Akt; however, both MK2 and p38 were detected in additional fractions in the absence of Akt and Hsp27. Furthermore, p38 MAPK was detected in some fractions in the absence of Akt, Hsp27, and MK2. These results suggest that under control conditions, almost 100% of Hsp27 and Akt associate with MK2, whereas a small percentage of MK2 exists in a complex with p38 MAPK in the absence of Akt and Hsp27 (Fig. 2A). These results

FIGURE 1. Enhanced Akt/Hsp27 interaction during LPS delay of apoptosis. Lysates from human PMNs freshly isolated or in culture for 24 h in the presence and absence of LPS (100 ng/ml) were subjected to anti-Akt immunoprecipitation (IP) and immunoblotted (IB) with anti-Hsp27 antibody. Lysates from freshly isolated PMNs were also subjected to isotype control immunoprecipitation as control. As a control, immunoprecipitates were immunoblotted with anti-Akt antibody to demonstrate equal amounts of immunoprecipitations in each condition.

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were confirmed by performing Akt immunoprecipitations and immunoblotting with anti-Hsp27 and anti-MK2 antisera (data not shown). In fMLP-stimulated PMNs, we demonstrated that Hsp27 eluted out prior to Akt (Fig. 2B). Thus, only a small fraction of Hsp27 associates with Akt, whereas a large amount of Hsp27 was not in a complex with Akt in fMLP-stimulated PMNs. MK2 and p38 MAPK continue to associate with Akt after fMLP stimulation.

**Phosphorylation Induces an Acidic Shift in Hsp27 pI without Altering Its Molecular Size in Human PMNs**—The oligomerization status of Hsp27 in human PMNs has not been demonstrated to date. To determine whether Hsp27 existed at different isoelectric points by the virtue of its phosphorylation, control and fMLP-stimulated PMN lysates were subjected to IEF gel electrophoresis and immunoblotting with anti-Hsp27 antibody. The results demonstrated two species of Hsp27 with different isoelectric points in both control and fMLP-stimulated PMNs. MK2 and p38 MAPK continue to associate with Akt after fMLP stimulation.

In fMLP-stimulated PMNs, we demonstrated that Hsp27 eluted out prior to Akt (Fig. 2B). Thus, only a small fraction of Hsp27 associates with Akt, whereas a large amount of Hsp27 was not in a complex with Akt in fMLP-stimulated PMNs. MK2 and p38 MAPK continue to associate with Akt after fMLP stimulation.

**Regulation of Akt Activation by Hsp27**

![Figure 2](image1.png)

**Figure 2.** Separation of Akt signal components in control and fMLP-stimulated PMNs. Human PMN lysates generated from control (A) and 0.3 μM fMLP-treated (B) PMNs were subjected to gel filtration chromatography. Eluted protein fractions were collected as described under “Materials and Methods.” Protein fractions were subjected to SDS-PAGE and immunoblotting with anti-Akt, anti-Hsp27, anti-MK2, and anti-p38 MAPK antisera. Results show that Akt and Hsp27 elute out in the same fractions in control PMN lysates, although a majority of Hsp27 elutes out prior to Akt in fMLP-treated PMN lysates, suggesting its dissociation from Akt. MK2 and p38 MAPK associate with Akt in the presence and absence of fMLP.

![Figure 3](image2.png)

**Figure 3.** Phosphorylation induces an acidic shift in Hsp27 pI without altering its molecular size in human PMNs. A, human PMN lysates generated from control (C) and 5-min fMLP (F5) (0.3 μM)-treated PMNs were subjected to IEF electrophoresis and immunoblotted (IB) with anti-Hsp27 and anti-Ser(P)-82Hsp27 antisera as described under “Materials and Methods.” IEF immunoblotting demonstrated two species of Hsp27 with different isoelectric points in both control and fMLP-stimulated PMN lysates. Increased abundance of the more acidic species of Hsp27 was seen in fMLP-treated lysates. Immunoblot analysis with anti-Ser(P)-82Hsp27 antibody confirmed that the increase in the acidic form of Hsp27 correlated with enhanced Hsp27-Ser82 phosphorylation. Phosphorylation of Hsp27 induced an acidic pI shift of Hsp27 without altering its size in human PMNs.

![Two-Dimensional PAGE](image3.png)

**Figure 3.** Two-dimensional polyacrylamide gels were subsequently subjected to immunoblotting with anti-Hsp27 antibody. Results demonstrated two species of Hsp27; however, both these species were of identical size. Collectively, these results demonstrate that phosphorylation of Hsp27 induced an acidic pI shift of Hsp27 without altering its size in human PMNs.
PMNs results in loss of Akt activation and PMN apoptosis (8). To determine whether loss of Akt phosphorylation and activation in the absence of Hsp27 results from loss of interaction between Akt and its upstream activator kinase MK2, PMNs were incubated in the presence or absence of anti-Hsp27 antibody or isotype control antibodies as described previously (8). Successful introduction of FITC-tagged antibodies into PMNs was confirmed by confocal microscopy and trypan blue quenching as described previously by our group (8). After 4 h, appropriate antibody incubation cell lysates generated were subjected to isotype control or anti-Akt immunoprecipitation (IP) followed by immunoblot (IB) analysis with anti-Akt antibody. Results demonstrate Akt/Hsp27 interaction in isotype control-treated PMNs but not in anti-Hsp27 treated PMNs (n = 3). PMN lysates obtained above were incubated with GST or GST-Akt-Sepharose. Proteins were separated by SDS-PAGE and immunoblotted with anti-MK2 antibody (n = 3). Isotype control-treated PMN lysate was included as a positive control for MK2 (5th lane). Results show that depletion of Hsp27 inhibits Akt/MK2 interaction. C. PMNs were treated with mouse isotype control antibody or monoclonal anti-Hsp27 antibody for 4 h at 37 °C. Isotype control and anti-Hsp27 antibody-treated cells were stained with FITC-conjugated annexin V and viewed by confocal microscopy. Annexin V-positive staining was observed in anti-Hsp27 antibody-treated cells.

FIGURE 4. Hsp27 depletion induces PMN apoptosis by inhibiting Akt association with its upstream kinase MK2. A, human PMNs were treated with isotype control (IC) antibody or Hsp27 antibody for 4 h at 37 °C as described previously (8). PMN lysates were subjected to anti-Akt or isotype control immunoprecipitation (IP) followed by immunoblot (IB) analysis with anti-Akt antibody. Results demonstrate Akt/Hsp27 interaction in isotype control-treated PMNs but not in anti-Hsp27 treated PMNs (n = 3). PMN lysates obtained above were incubated with GST or GST-Akt-Sepharose. B, proteins were separated by SDS-PAGE and immunoblotted with anti-MK2 antibody (n = 3). Isotype control-treated PMN lysate was included as a positive control for MK2 (5th lane). Results show that depletion of Hsp27 inhibits Akt/MK2 interaction. C. PMNs were treated with mouse isotype control antibody or monoclonal anti-Hsp27 antibody for 4 h at 37 °C. Isotype control and anti-Hsp27 antibody-treated cells were stained with FITC-conjugated annexin V and viewed by confocal microscopy. Annexin V-positive staining was observed in anti-Hsp27 antibody-treated cells.
Regulation of Akt Activation by Hsp27

Silencing Hsp27 expression disrupts Akt/MK2 interaction. HK-11 cells were transfected with scrambled or Hsp27 siRNA. A, cell lysates from transfected cells were immunoblotted (IB) with anti-Hsp27 and anti-Akt antisera to demonstrate specific silencing of Hsp27 expression (n = 3). B, above lysates were subjected to GST or GST-Aktwt pulldown assay to determine Akt/MK2 interaction in the presence of scrambled or Hsp27 siRNA (n = 3). C, above lysates were also subjected to mouse anti-Akt immunoprecipitation (IP) and immunoblotted with rabbit polyclonal anti-MK2 and rabbit polyclonal anti-Akt antisera (n = 3). The results demonstrate loss of Hsp27 expression abrogates Akt/MK2 interaction.

Disruption of Akt/MK2 Interaction by Silencing Hsp27 Expression Induces HK-11 Cell Death—Silencing Hsp27 expression inhibits Akt/MK2 interaction and Akt activation. Thus, we determined effects of silencing Hsp27 expression on HK-11 cell viability.

HK-11 cells alone or HK-11 cells in the presence of GenePORTER reagent, scrambled siRNA, or Hsp27 siRNA were incubated at 37 °C in 5% CO2 for 24 h, and cells were subjected to MTT cell viability assay. Hsp27 siRNA but not scrambled siRNA significantly inhibited Hsp27 without altering Akt expression (Fig. 7, bottom panel). Additionally, Hsp27 siRNA but not scrambled siRNA significantly decreased HK-11 cell viability. GenePORTER reagent by itself had no effect on cell viability (Fig. 7, top panel). These results suggest that Hsp27 is a survival protein, and its regulation of HK-11 survival may be mediated by scaffolding MK2 to Akt, allowing Akt activation to occur, thereby promoting cell survival.

Akt PH Domain Is Not Required for Interaction with Hsp27—We have demonstrated previously that Hsp27 directly interacts with Akt (8). To identify Hsp27-binding site(s) on Akt, we determined the ability of GST-Akt-(1–149)-Sepharose, which contains the Akt PH domain (1–116 amino acids) and the acidic linker region (117–149) to interact with recombinant Hsp27. Recombinant GST-Akt-(1–149) was conjugated to glutathione-Sepharose as described under “Materials and Methods.” Fig. 8A demonstrates that GST-Akt-(1–149) (2nd lane), but not glutathione-Sepharose (1st lane), specifically interacts with recombinant Hsp27 suggesting a role for the PH domain and/or the acidic linker region to interact with Hsp27. Recombinant GST-Akt-(1–149) and recombinant Hsp27 were run as positive controls (Fig. 8A, 3rd and 4th lanes). To determine the role of Akt PH domain in interacting with Hsp27, recombinant AktΔPH (mutant lacking N-terminal PH domain or 1–116 amino acids) was precipitated with GST or GST-Akt. Fig. 8B demonstrates interaction of recombinant AktΔPH with GST-Hsp27 but not with GST-Sepharose. Recombinant AktΔPH was run as positive control (Fig. 8B, lane 3). These results suggest that Akt-PH domain is not required for its interaction with Hsp27.
Regulation of Akt Activation by Hsp27

Hsp27 Directly Interacts with the Acidic Linker Region of Akt—In-frame deletion mutant of Akt was generated such that amino acids 117–128 from the acidic linker region of Akt were deleted. In a GST pulldown assay recombinant Hsp27 was precipitated by GST-Aktwt but not by GST or GST-AktΔ117–128-Sepharose (Fig. 9A, bottom panel). Transferred gel was stained with Coo massaie Blue to serve as a loading control for GST beads utilized in the assay. Additionally, HEK-293 cells were co-transfected with c-Myc-tagged pUseAktwt or pUseAktΔ117–128 along with Hsp27 cDNA. After transfection cells were lysed, and lysates were subjected to anti-c-Myc immunoprecipitation and immunoblotted with anti-Hsp27 antibody. Fig. 9B (bottom panel) demonstrates interaction between Aktwt and Hsp27, whereas no interaction is detected between AktΔ117–128 and Hsp27. To demonstrate overexpression of c-Myc-tagged Akt constructs and Hsp27, total lysates were immunoblotted with anti-c-Myc and anti-Hsp27 antisera (Fig. 9B, top and middle panel). Furthermore, these results were confirmed in human PMNs by performing a GST pulldown assay. GST-Akt but not GST or GST-AktΔ117–128 interacted with Hsp27 from PMN lysates (Fig. 9C). Transferred gels were stained with Coo massiae Blue to demonstrate equal loading of GST-Akt and GST-AktΔ117–128-Sepharose. These results collectively indicate that amino acids 117–128 in the acidic linker region of Akt are required for interaction with Hsp27.

We next determined the ability of GST, GST-Aktwt, and GST-AktΔ117–128 to interact with Hsp90 from PMN lysates. Hsp90 has been shown previously to directly interact with Akt via amino acids 229–309 (38). To confirm that deletion of 117–128 amino acids from Akt did not alter protein conformation and its ability to interact with Hsp90, we subjected GST, GST-Akt, and GST-AktΔ117–128 to a GST pulldown assay with PMN lysate. Fig. 9D demonstrates that as expected both GST-Aktwt and GST-AktΔ117–128 but not GST interacted with Hsp90 from PMN lysates. PMN lyase run as positive control immunoreacted with anti-Hsp90 antibody (Fig. 9D, lane 1). Transferred gels were stained with Coo massiae Blue to demonstrate equal loading of GST-Akt and GST-AktΔ117–128-Sepharose. These results collectively indicate that Hsp27 interacts with Akt via amino acids 117–128, and the deletion of this region does not severely alter its conformation and binding to its known binding protein Hsp90.

MK2 Fails to Interact with AktΔ117–128 and Phosphorylate It on Ser-473 ex Vivo—Having disrupted Akt/Hsp27 interaction by deleting Hsp27 binding region on Akt, we next determined the ability of GST, GST-Aktwt, and GST-AktΔ117–128 to precipitate MK2 from PMN lysates. Fig. 10A shows that GST-Aktwt, but not GST or GST-AktΔ117–128, interacted with MK2 from PMN lysate. Transferred gels were stained with Coo massiae Blue to demonstrate equal loading of GST-Akt and GST-AktΔ117–128-Sepharose. Having shown loss of MK2 binding to AktΔ117–128, we next determined the ability of constitutively activated MK2 (MK2EE), a known upstream activator of Akt, to phosphorylate Aktwt and AktΔ117–128 in transfected HEK-293 cells. Fig. 10B shows that MK2EE phosphorylates Aktwt on Ser-473 but fails to phosphorylate AktΔ117–128. These results establish a novel role for Hsp27 in regulating Akt activation by scaffolding MK2 to Akt signal complex.
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![Image of a gel blot with Coomassie Blue staining showing the expression of Akt and Hsp27.](image)

**FIGURE 9.** Hsp27 directly interacts with the acidic linker region of Akt. A, GST, GST-Aktwt, or GST-Akt<sub>Δ117-128</sub> (117–128 amino acids deleted) was subjected to a pulldown assay with recombinant Hsp27 (50 ng). Transferred gel was stained with Coomassie Blue to serve as a loading control of GST beads utilized in the assay. Recombinant Hsp27 associated with GST-Aktwt but failed to bind GST or GST-Akt<sub>Δ117-128</sub>-Sepharose. B, HEK-293 cells were transfected with Hsp27 along with either c-Myc-tagged Aktwt or in-frame Akt deletion mutant Akt<sub>Δ117-128</sub> (117–128 amino acids deleted). Transfected lysates were immunoprecipitated (IP) with anti-c-Myc antibody and immunoblotted (IB) with anti-Hsp27 antibody (middle panel). Transfected lysates were also immunoblotted with anti-Hsp27 and anti-c-Myc antibodies to demonstrate expression of Hsp27 and c-Myc-tagged Akt constructs (top and bottom panels). C, PMN lysate was incubated with GST, GST-Akt, and GST-Akt<sub>Δ117-128</sub> and subjected to a GST pulldown assay and immunoblotted with anti-Hsp27 antibody (n = 3). Transferred gels were stained with Coomassie Blue to demonstrate equal loading of GST-Akt and GST-Akt<sub>Δ117-128</sub>-Sepharose. D, PMN lysate was incubated with GST, GST-Akt, and GST-Akt<sub>Δ117-128</sub> and subjected to a GST pulldown assay and immunoblotted with anti-Hsp90 antibody (n = 3). Transferred gels were stained with Coomassie Blue to demonstrate equal loading of GST-Akt and GST-Akt<sub>Δ117-128</sub>-Sepharose. Results show that Akt<sub>Δ117-128</sub> mutant binds Hsp90 but fails to interact with Hsp27.

**DISCUSSION**

The first indication that Hsp27 might regulate Akt activity was reported by Konishi *et al.* (39), who demonstrated an association of Hsp27 and Akt in COS-7 cells. Subsequently, we demonstrated for the first time that Akt exists in a signaling complex with p38 MAPK, MK2, and Hsp27 in human PMNs (3). More recently, p38 MAPK-dependent activation of Akt has been demonstrated in a variety of cells, including cardiomyocytes, keratinocytes, and endothelial cells (40–47). Thus, p38 MAPK regulation of Akt activation is not unique to PMNs. In this study we show that Hsp27 directly interacts with Akt through its acidic linker region. The physical association of Hsp27 with Akt is a critical determinant of PMN survival, as removal of Hsp27 from the Akt signal module prevents Akt activation and results in accelerated PMN apoptosis (8). Moreover, MK2 in addition to phosphorylating Hsp27 (29) acts as PDK2 for Akt in human PMNs, phosphorylating Ser-473 (3).

Based on these studies, we postulated that Hsp27 regulates PMN apoptosis by acting as a scaffolding protein in the Akt signaling complex. Hsp27 binds to p38 MAPK, MK2, and other proteins required for activation of Akt. In this study we show for the first time relevance of Akt/Hsp27 interaction to constitutive apoptosis and LPS delay of apoptosis. Akt/Hsp27 interaction was documented in freshly isolated PMNs and was markedly reduced in PMNs cultured for 24 h, a time point at which only about 30% of PMNs are viable in culture (2). In contrast, Akt-Hsp27 association was markedly enhanced in PMNs cultured for 24 h in the presence of LPS. We have shown previously that LPS delays constitutive PMN apoptosis with 70% viable PMNs at 24 h (2). In addition, we have shown that Hsp27 is a member of the Akt signal complex, and removal of Hsp27 from the complex results in loss of Akt activation and induction of PMN apoptosis. These findings indicate a role for Hsp27 in the regulation of inflammation by modulation of Akt activation and PMN apoptosis.

Isolation of Akt signal complex in the presence and absence of fMLP by gel filtration chromatography demonstrated that Hsp27 dissociated from Akt upon fMLP stimulation as demonstrated previously by our group (3). In addition, IEF gel electrophoresis/immunoblot analysis demonstrated an acidic pl shift in Hsp27 in fMLP-stimulated PMNs compared with control, coinciding with Hsp27Ser-82 phosphorylation. Moreover, two-dimensional PAGE immunoblotting demonstrated that this acidic pl shift in Hsp27 occurred without altering the molecular size of Hsp27. Thus, phosphorylation of Hsp27 does not alter the molecular size of Hsp27 in PMNs. Therefore, Hsp27 dissociation from Akt upon PMN activation is dependent on Hsp27 phosphorylation and not on the molecular size of Hsp27.

Heat shock proteins are induced under various stress conditions to combat stress-induced apoptosis caused by aggregation of denatured proteins. Heat shock proteins bind to and refold denatured proteins and chaperone them to appropriate cellular compartments or target them to the proteasome for degradation. Hsp27 binds actin and stabilizes the cellular architecture (48). The protective effect of Hsp27 on the cytoskeleton is mediated by the ability of Hsp27 to bind denatured actin and prevent its aggregation (49). Arigo *et al.* (50) demonstrated...
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Akt, Hsp27 and MK2EE (constitutively active MK2) along with either Aktwt or AktCA in the presence and absence of Hsp27 siRNA. Immunoblotting studies demonstrated that loss of Hsp27 expression inhibited AktSer473 phosphorylation and activation. These studies clearly identified a role for Hsp27 in regulating Akt/MK2 interaction and Akt activation. Cell death induced by silencing Hsp27 was not entirely surprising as Schepers et al. (58) have shown that silencing Hsp27 expression resulted in a 2-fold increase in VP-17-induced apoptosis in acute myeloid leukemia cells. However, induction of cell death by modulating Akt/MK2 interaction was a novel observation. To determine more directly the role of Hsp27 in regulating Akt activation, HK-11 cells were transfected with myristoylated-c-Myc-tagged Akt (AktCA) in the presence and absence of Hsp27 siRNA. Immunoblotting studies demonstrated that loss of Hsp27 expression inhibited AktSer473 phosphorylation and activation. These studies clearly identified a role for Hsp27 in regulating Akt activation. Hsp27 negatively regulates protein kinase Cδ activity and inhibits cell apoptosis (59); however, we show for the first time that Hsp27 positively regulates Akt activation and promotes cell survival.

We have demonstrated direct protein/protein interaction between Hsp27 and Akt (3, 8). This study identified amino acids 117–128 in the acidic linker region of Akt to mediate interaction with Hsp27. An in-frame Akt deletion mutant (AktΔ117–128) interacted with Hsp90α but failed to interact with Hsp27 and MK2. This finding is in accordance with the literature, which demonstrated that Akt interacted with Hsp90α via amino acids 229–309 (38).

These studies suggested that loss of AktΔ117–128 interaction with MK2 and Hsp27 was not because of altered conformation of the mutant. The importance of Hsp27 in regulating Akt/MK2 interaction and AktSer473 phosphorylation was documented by co-transfecting HEK-293 cells with constitutively active MK2 (MK2EE) and Hsp27 along with Aktwt or AktΔ117–128-MK2EE phosphorylated Aktwt on Ser473 as expected; however, it failed to phosphorylate AktΔ117–128 on Ser473, a critical site known to regulate Akt activation (60). Furthermore, the physiologic consequence of loss of Akt/MK2 interaction and loss of Ser473 phosphorylation resulted in cellular apoptosis. These studies collectively define a novel role of Hsp27 in regulating Akt activation and apoptosis by mediating interaction between Akt and its upstream activator MK2.

A

FIGURE 10. MK2 fails to interact with AktΔ117–128 and phosphorylate it on Ser473 ex vivo. A, PMN lysate was incubated with GST, GST-Akt, and GST-AktΔ117–128 and subjected to a GST pulldown assay and immunoblotted with anti-MK2 antibody (n = 3). Transferred gels were stained with Coomassie Blue to demonstrate equal loading of GST-Akt and GST-AktΔ117–128-Sepharose. Results show that AktΔ117–128, mutant fails to interact with MK2 from PMN lysates. B, HEK-293 cells were transfected with vector or co-transfected with Hsp27 and MK2EE (constitutively active MK2) along with either Aktwt or AktΔ117–128. Transfected lysates were immunoblotted with anti-Hsp27, anti-MK2, anti-c-Myc, and anti-pAktS473 antisera. Results show that active MK2 fails to phosphorylate AktΔ117–128 ex vivo in HEK 293 cells.

that Hsp27 inhibited apoptosis by maintaining the redox equilibrium of the cell. In addition, overexpression of Hsp27 inhibited oxidant stress-induced cellular damage by increasing levels of cellular glutathione (51). Overexpression of various heat shock proteins has been shown to induce a survival response. Contrary to most heat shock proteins, Hsp60 (52, 53) has been shown to induce apoptosis by interaction with caspase-3, whereas Hsp27 (21–25), Hsp70 (54–56), and Hsp90 (57) have been shown to inhibit apoptosis by binding to and inactivating specific components of the apoptosis machinery. In general heat shock protein-induced protection from stress was shown to act at the level of the apoptosome by preventing downstream activation of caspases. This study defines a novel role of Hsp27 in the regulation of cellular apoptosis by acting as a scaffolding protein.

We have recently demonstrated that Hsp27 exists in a complex with Akt, p38 MAPK, MK2, and Hsp27, and MK2 acts as PDK2 for Akt (3). In addition, we demonstrated that MK2-binding protein Hsp27 dissociated from Akt signal complex after Akt activation (3). However, disruption of Akt/Hsp27 interaction prior to Akt activation resulted in loss of fMLP-stimulated Akt activation and induced PMN apoptosis (8). These studies indicated the importance of Akt/Hsp27 interaction to Akt activation and PMN survival. Recently Zheng et al. (30) demonstrated that MK2 was required for p38 MAPK/Hsp27 interaction. However, MK2 was not required for association of Hsp27 with Akt. Given that Akt/Hsp27 interaction was not regulated by MK2 and that MK2 serves as PDK2 for Akt, we hypothesized that Hsp27 modulated interactions within the Akt-Hsp27-MK2 signal complex by acting as a scaffolding protein. This study defines a novel scaffolding role of Hsp27 in the regulation of Akt activation and cellular apoptosis.

Disruption of Akt/Hsp27 interaction by depleting Hsp27 from human PMNs resulted in loss of Akt interaction with both Hsp27 and MK2 and induction of PMN apoptosis. Because MK2 is an Hsp27-binding protein, we next determined whether depletion of Hsp27 nonspecifically removed MK2 from the Akt signal complex. To address this question, siRNA technology was utilized to specifically silence Hsp27 expression from HK-11 cells to determine effects of silencing Hsp27 on Akt/MK2 interaction and HK-11 cell viability. Hsp27 siRNA but not scrambled siRNA-transfected HK-11 cells demonstrated a marked decrease in Hsp27 expression, loss of Akt/MK2 interaction, and induction of HK-11 cell death. These results suggested a role for Hsp27 in regulating Akt/MK2 interaction and Akt activation. Cell death induced by silencing Hsp27 was not entirely surprising as Schepers et al. (58) have shown that silencing Hsp27 expression resulted in a 2-fold increase in VP-17-induced apoptosis in acute myeloid leukemia cells. However, induction of cell death by modulating Akt/MK2 interaction was a novel observation. To determine more directly the role of Hsp27 in regulating Akt activation, HK-11 cells were transfected with myristoylated-c-Myc-tagged Akt (AktCA) in the presence and absence of Hsp27 siRNA. Immunoblotting studies demonstrated that loss of Hsp27 expression inhibited AktSer473 phosphorylation and activation. These studies clearly identified a role for Hsp27 in regulating Akt activation. Hsp27 negatively regulates protein kinase Cδ activity and inhibits cell apoptosis (59); however, we show for the first time that Hsp27 positively regulates Akt activation and promotes cell survival.
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