Hsp27 Promotes Insulin-Like Growth Factor-I Survival Signaling in Prostate Cancer via p90Rsk-Dependent Phosphorylation and Inactivation of BAD

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Abstract

Hsp27 is highly expressed in castrate-resistant prostate cancer. Although its overexpression confers resistance to androgen ablation and chemotherapy, the mechanisms by which Hsp27 inhibits treatment-induced apoptosis are incompletely defined. Castrate-resistance often correlates with increased activity of autocrine and/or paracrine growth/survival stimulatory loops including the mitogen-activated protein kinase (MAPK) and Akt pathways and insulin-like growth factor (IGF) axis components. Because Hsp27 can be activated by both MAPK and Akt pathways, it is possible that interactions between IGF-I signaling and Hsp27 phosphoactivation function to promote castrate-resistant progression. Here, we report that Hsp27 expression and phosphorylation levels correlate with IGF-I signaling and castrate-resistant progression in human prostate cancer specimens and cell lines. IGF-I induces Hsp27 phosphorylation in a time- and dose-dependent manner via p90Rsk, which interacts directly with and phosphorylates Hsp27 in vitro and in vivo. Conversely, p90Rsk inhibition using short interfering RNA or a dominant negative mutant abolishes IGF-I-induced Hsp27 phosphorylation. Hsp27 overexpression increases IGF-I-induced phosphorylation of Erk, p90Rsk, and Akt. Conversely, Hsp27 knockdown abrogates IGF-I-induced phosphorylation of Erk, p90Rsk, and Akt, thereby destabilizing Bad/14-3-3 complexes and increasing apoptotic rates. These data elucidate the interactions between Hsp27 phosphorylation and the IGF-I receptor signaling pathway and support targeting Hsp27 as a therapeutic strategy for castrate-resistant prostate cancer.

Introduction

Prostate cancer is the second leading cause of cancer deaths in North American men, killing more than 40,000 annually. A major challenge for the treatment of men with metastatic or recurrent disease is the development of treatment resistance after androgen deprivation therapy even when combined with docetaxel chemotherapy. Although the majority of patients initially respond to such treatments, most go on to develop lethal castrate-resistant prostate cancer (CRPC). Under conditions of castrate levels of serum testosterone, other growth factors assume a more dominant role in promoting cancer cell survival (1). CRPC is a complex process involving clonal selection (2) and androgen receptor (AR) activation by low levels of androgens, receptor mutations, or increased coactivator levels (3–5). Additional mechanisms include the activation of alternative growth factor pathways such as insulin-like growth factor (IGF; refs. 6, 7) and upregulation of antiapoptotic genes including Hsp27 (8).

The IGF axis is an important regulator of growth, survival, and metastatic potential in a variety of malignancies and is strongly implicated in prostatic carcinogenesis and CRPC (9, 10). IGF-I mediates growth and survival responses by binding to the tyrosine kinase IGF-I receptor (IGF-IR). Once activated, IGF-IR phosphorylates the insulin receptor substrate, which leads to the activation of many signaling pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK) or PI3K/Akt. Activation of PI3K/Akt by IGF-I leads to Bad phosphorylation on Ser136 and the activation of forkhead-regulated transcription factor (11). Activation of Erk phosphorylates p90 ribosomal S6 kinase (p90Rsk) which, in turn, leads to the phosphorylation of Bad on Ser112 (12). Hence, the activation of PI3K and Erk pathways converge to inhibit apoptosis by phosphorylating (on Ser136 and Ser112, respectively) and inactivating the proapoptotic protein Bad.

Hsp27 is a stress-induced molecular chaperone that is highly and uniformly expressed in CRPC (13, 14) and other cancers (15–17), and is often associated with metastasis and poor prognosis (18, 19). Hsp27 expression is induced by hormone therapy or chemotherapy and inhibits treatment-induced apoptosis through multiple mechanisms (8, 18,
Hsp27 has been shown to interact and inhibit components of both stress-induced and receptor-induced apoptotic pathways. Hsp27 prevents the activation of caspases by sequestering cytochrome c in the cytoplasm (23). Cytochrome c interacts with Apaf-1 and caspase-9 to form the “apoptosome”, which activates caspase-3 and a cascade of downstream caspases, the so-called “effectors” of cell death (24). Hsp27 interacts with and inhibits caspase-3 activation, an effect related to the ability of Hsp27 to stabilize actin microfilaments (25). Hsp27 binds to filamentous actin to prevent the disruption of the cytoskeleton resulting from either heat shock and other stresses (26), and also inhibits the mitochondrial release of Smac to confer resistance to dexamethasone in multiple myeloma cells (27). Hsp27 is also involved in the regulation of AKT, inhibiting Bax activation to enhance cell survival (28). Hsp27 could inhibit apoptosis induced by etoposide or tumor necrosis factor-α by increasing IkBa ubiquitination and degradation, which increases the activity of the survival transcription factor nuclear factor-κB (29).

Although both increased IGF-I/IGF-IR signaling and Hsp27 expression are associated with CRPC progression (6, 7, 30, 31), the precise mechanisms by which the prosurvival effects of IGF-I/Hsp27 drive CRPC progression are incompletely defined. Here, we define the links between Hsp27 phosphorylation and IGF-1 signaling in CRPC, identifying p90Rsk as a downstream effector of IGF-1-activated Erk that directly phosphorylates Hsp27 as a key step in the inhibition of Bad/14-3-3 complex formation and apoptosis.

Materials and Methods

**Plasmids.** Hsp27 wild-type was subcloned into lentivirus vector pHF-cytomegalovirus as previously described (8). Hsp27 triple mutant alanine (Ala) was generated by direct mutagenesis replacing Ser7, Ser17, and Ser82, respectively, with alanine using QuikChange mutagenesis kit according to the instructions of the manufacturer (Stratagene). p90Rsk wild-type and dominant negative (DN) plasmids were generously provided by Dr. J. Blenis (Harvard University, Boston, MA).

**Antibodies and reagents.** Hsp27-1 was purchased from Research Diagnostics, Inc. SB 203580, Ro 31-8220, PD 98056, and cycloheximide were from Calbiochem. Total Hsp27/pHsp27 was from Assay Designs; pAkt/Akt, Bad, total Erk, and poly(ADP ribose)polymerase were from Cell Signaling Technology; p90Rsk/p-p90Rsk, pErk, p38 kinase, AR, IGF-IR, 14-3-3 antibodies, and p90Rsk short interfering RNA (siRNA) were from Santa Cruz Biotechnology, Inc. Purified recombinant nonphosphorylated Hsp27 (expressed in bacteria) and active p90Rsk (expressed in SE21 cells) were purchased from Assay Designs and Upstate Biotechnology, respectively. Secondary antibodies FITC and Rhodamine Red-x were purchased from Jackson ImmunoResearch Laboratories, Inc., and Flag-Agarose was purchased from Sigma Aldrich.

**Animal manipulation.** All animal experimentation was conducted in accordance with accepted standards of the University of British Columbia Committee on Animal Care. LNCaP and PC-3 xenograft tumors were grown in athymic nude mice at four sites as modified from a previously reported method (5). Prostate specific antigen levels were measured weekly by tail vein sera samples using an immunoassay kit (ClimPro). Mice were castrated at 6 wk postinoculation with LNCaP cells. Tumors were harvested from the same mouse precastration (intact), and at 35 d postcastration (CRPC). Tumors were excised, dissected, and immediately frozen in liquid nitrogen.

**Immunohistochemistry.** A tissue microarray was constructed using a Beecher microarray from paraffin-embedded specimens with matching H&E slides. Each case was represented with three cancer cores in the tissue microarray analysis. Sections were deparaffinized and rehydrated with xylene and ethanol, and then transferred to 0.02% triton for permeabilization. Slides in citrate buffer (pH 6) were heated in the steamer for 30 min, washed for 5 min in PBS, incubated in 3% bovine serum albumin transferred to 3% H2O2, and incubated overnight with anti-pHsp27 antibodies (Cell Signaling) as described (8).

**Cell proliferation assay.** PC-3 cells were plated in DMEM with 5% fetal bovine serum. The next day, serum-free medium was used with or without 50 ng/mL of IGF-I and cell proliferation was done (32).

**Western blot analysis.** PC-3 cells were first serum-starved overnight in DMEM. IGF-I was then added at different concentrations or at different times. Inhibitors were preincubated for 1 h prior to the addition of IGF-I. After treatment, cells were lysed in radioimmunoprecipitation assay buffer and submitted to Western blot as previously described (32).

**Kinase assay.** One microgram of recombinant Hsp27 was incubated at 30°C for 1 h with different concentrations of the purified active form of p90Rsk in reaction buffer [50 mmol/L Hepes (pH 7.4), 10 mmol/L MgCl2, 2308 Cancer Res; 70(6) March 15, 2010 Cancer Research
1 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L β-mercaptoethanol, 0.5 mmol/L vanadate, 50 μmol/L ATP, and 5 uCi of 32P-γ-ATP. The p90RSK inhibitor (Ro-31-8220) was added to show the specificity of the reaction.

**Immunoprecipitation.** Total proteins (500 μg) were pre-cleared with protein-G sepharose (Invitrogen-Life Technologies, Inc.) for 1 h at 4°C and immunoprecipitated with 2 μg of anti-Bad overnight at 4°C. The immune complexes were recovered with protein-G sepharose for 2 h and then washed with radioimmunoprecipitation assay buffer at least three times, centrifuged, and submitted to SDS-PAGE followed by Western blotting. For p90RSK pulldown, cells were lysed in radioimmunoprecipitation assay buffer 48 h after transfection, and 750 μg of protein were incubated with Flag-Agarose for 3 h; the immune complexes were washed and submitted to Western blotting.

**Caspase-3 activity.** PC-3 cells were treated once daily for 2 d with 10 nmol/L of siRNA or Scramble control. After the second transfection, cells were incubated with IGF-I for 48 h. Caspase-3 assay was performed using the manufacturers recommendations (BIOMOL Research Laboratories, Inc.). Briefly, 50 μg of total proteins were assessed to cleave the Ac-DEVD-AMC substrate. The cleaved products yielded a fluorogenic reaction and were read in a microplate reader using a filter with a 360 nm excitation/460 emission wavelength.

**Immunofluorescence.** PC-3 cells were grown on coverslips and fixed in paraformaldehyde for 10 min at room temperature and immunofluorescence was performed as described previously (32) using Hsp27 (1:500) and p90RSK (1:100) antibodies.

**Flow cytometry analysis.** PC-3 cell lines were plated in 10 cm dishes and 24 h later, cells were serum-starved and treated with or without 50 μg/mL of IGF-I or 10 μg/mL of cycloheximide for 24 h. Sub-G0 was analyzed as described previously in ref. (32).

**Statistical analysis.** All data were analyzed by Student’s t test. Levels of statistical significance were set at P < 0.05 (two-sided), and all statistical calculations were done using StatView 4.5 (Abacus Concepts, Inc.).

**Results**

Phosphorylated Hsp27 is highly expressed in human CRPC tumors. To characterize the levels of both total and phosphorylated Hsp27 (p-Hsp27) levels with CRPC, we analyzed staining patterns in human prostate cancer tissues before and after androgen ablation therapy. Figure 1A illustrates that total and p-Hsp27 levels are low in untreated tumors but the staining intensity increases after androgen ablation, becoming uniformly strong in CRPC and statistically higher compared with untreated patients (Fig. 1B). The increased levels of p-Hsp27 correlated with total Hsp27. We next characterized changes in total and phosphorylated Hsp27 levels in the human LNCaP xenograft model, which mimics the progression to CRPC after castration. Western blot analysis of treatment-naive and CRPC LNCaP tumors showed that p-Hsp27 levels increased after castration with no significant difference in total Hsp27 levels (Fig. 1C). This increase in p-Hsp27 levels correlated with increased levels of phosphorylated (but not total) Erk/p90RSK and Akt protein levels (Fig. 1C). Increased p-Hsp27 levels also correlated with increased prostate serum antigen levels, suggesting a correlation between Hsp27 phosphorylation and AR-regulated prostate serum antigen expression as reported previously (32). Next, we evaluated p-Hsp27 levels in androgen-responsive LNCaP cells, castrate-resistant C4-2 cells, and AR-negative PC-3 cells. Western blot analysis indicates that whereas total Hsp27 levels are slightly higher in PC-3 xenografts, p-Hsp27 levels are highly upregulated in PC-3 tumors compared with treatment-naive LNCaP tumors (Fig. 1D). Hsp27 expression and phosphorylation levels were also higher in C4-2 compared with LNCaP cells (data not shown). Collectively, these data indicate that increased p-Hsp27 levels correlate with CRPC.

IGF-I increases PC-3 cell proliferation. We selected PC-3 cells to study the effect of IGF-I on cell proliferation and survival because PC-3 cells are IGF-IR positive, AR negative, and express high basal levels of p-Hsp27 (Fig. 2A). IGF-I increased proliferative (Fig. 2B, left) and decreased apoptotic (Fig. 2B, right) rates in PC-3 cells grown in serum-free medium. Next, we investigated the effect of IGF-I on PC-3 cell survival using the apoptotic inducer cycloheximide. As expected, cycloheximide treatment increased cleaved poly(ADP ribose) polymerase (Fig. 2C, left) and sub-G0 population (Fig. 2C, right) as indicators of apoptosis, which were both reduced in the presence of IGF-I. Together, these results indicate that IGF-I is cytoprotective in PC-3 cells.

**IGF-I induces Hsp27 phosphorylation.** To identify the relationships between Hsp27 in IGF-I signaling and cell survival
in prostate cancer, we next determined if IGF-I phosphoactivates Hsp27. A dose-dependent and time course analysis of p-Hsp27 after IGF-I treatment was studied in parallel with the activation of IGF-IR signaling pathway. PC-3 cells were serum-starved overnight and treated with increasing concentrations of SB 203580 for 1 h prior to 10 min of IGF-I stimulation. Western blots were performed using antibodies for phosphorylated or total Hsp27 or p38 kinase. A, Akt inhibition does not affect IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with 20 μmol/L of LY 203580 for 1 h prior to 5 min of IGF-I stimulation. Western blots were performed using p-Hsp27, and p-Akt or T-Hsp27, or T-Akt for control loading. B, Akt inhibition does not affect IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with the indicated concentrations of PD 90059 for 1 h prior to 10 min of IGF-I stimulation. Proteins were extracted and Western blot performed using p-Hsp27, and p-Erk, or T-Hsp27 for control loading. C, Erk kinase inhibitor blocks IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with the indicated concentrations of PD 90059 for 1 h prior to 10 min of IGF-I stimulation. Proteins were extracted and Western blot performed using p-Hsp27, and p-Erk, or T-Hsp27 for control loading. D, effect of p90Rsk inhibition on IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with the indicated concentrations of Ro31-8220 for 1 h prior to 10 min of IGF-I stimulation. Proteins were extracted and Western blot performed using p-Hsp27/T-Hsp27 and p-p90Rsk/p90Rsk.

**Figure 3.** IGF-I phosphorylates Hsp27 downstream of MAPK. A, p38 kinase inhibition does not affect IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with the indicated concentrations of SB 203580 for 1 h prior to 10 min of IGF-I stimulation. Proteins were extracted and Western blots were performed using antibodies for phosphorylated or total Hsp27 or p38 kinase. B, Akt inhibition does not affect IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with 20 μmol/L of LY 203580 for 1 h prior to 5 min of IGF-I stimulation. Western blots were performed using p-Hsp27, and p-Akt or T-Hsp27, or T-Akt for control loading. C, Erk kinase inhibitor blocks IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with the indicated concentrations of PD 90059 for 1 h prior to 10 min of IGF-I stimulation. Proteins were extracted and Western blot performed using p-Hsp27, and p-Erk, or T-Hsp27 for control loading. D, effect of p90Rsk inhibition on IGF-I–mediated Hsp27 phosphorylation. PC-3 cells were serum-starved overnight and treated with the indicated concentrations of Ro31-8220 for 1 h prior to 10 min of IGF-I stimulation. Proteins were extracted and Western blot performed using p-Hsp27/T-Hsp27 and p-p90Rsk/p90Rsk.

In prostate cancer, we next determined if IGF-I phosphoactivates Hsp27. A dose-dependent and time course analysis of p-Hsp27 after IGF-I treatment was studied in parallel with the activation of IGF-IR signaling pathway. PC-3 cells were serum-starved overnight and treated with increasing concentrations of IGF-I. IGF-I potently induced dose- and time-dependent increases in Hsp27, Akt, and Erk phosphorylation (Fig. 2D, left). Interestingly, p-Hsp27 increased within 5 minutes after IGF-I exposure (Fig. 2D, middle), and as expected, IGF-I stimulated the time-dependent phosphorylation of p38 kinase, Akt, Erk, and p90Rsk. Moreover, IGF-I induced Bad/14-3-3 interaction in a time-dependent manner (Fig. 2D, right) as a consequence of Bad phosphorylation by both Akt and Erk pathways. These results confirm that IGF-I phosphoactivates Hsp27 and enhances Akt and Erk signaling in PC-3 prostate cancer cells.

**IGF-I leads to Hsp27 phosphorylation via the MAPK pathway.** To identify upstream effectors of IGF-I–induced Hsp27 phosphorylation, we pretreated PC-3 cells prior to IGF-I stimulation with selected specific inhibitors, SB 203580, LY 294002, and PD 90809 targeting p38 kinase, Akt, and Erk, respectively. The phosphorylation status of Hsp27, p38 kinase, Akt, and Erk were analyzed by Western blotting. Although neither SB203580 nor LY294002 altered IGF-I–induced Hsp27 phosphorylation (Fig. 3A and B), the Erk inhibitor PD 98059 abolished IGF-I–induced Hsp27 phosphorylation (Fig. 3C). We next tested whether IGF-I–induced Hsp27 phosphorylation occurs directly via Erk kinase or via its downstream effector, p90Rsk. PC-3 cells treated with the Ro-31-8220 inhibitor completely abolished IGF-I–induced Hsp27 phosphorylation (Fig. 3D), suggesting that IGF-I–mediated phosphorylation of Hsp27 occurs via the MAPK pathway downstream of Erk kinase.

**p90Rsk phosphorylates Hsp27 in vitro and in vivo.** To determine whether p90Rsk phosphorylates Hsp27, we performed an *in vitro* kinase assay using purified unphosphorylated Hsp27 and constitutively active p90Rsk. Figure 4A shows that in the absence of p90Rsk, Hsp27 is unphosphorylated and that only 50 ng of p90Rsk was sufficient to phosphorylate 1 μg of Hsp27 (1:50; Fig. 4A, left). Moreover, the p90Rsk inhibitor Ro-31-8220 significantly suppressed the ability of p90Rsk to phosphorylate Hsp27 (Fig. 5A, right), suggesting that p90Rsk specifically and directly phosphorylates Hsp27 *in vitro*. To corroborate this finding, we examined the ability of p90Rsk to phosphorylate Hsp27 *in vivo*. Overexpression of wild-type p90Rsk increased Hsp27 phosphorylation,
Figure 4. p90Rsk phosphorylates and interacts with Hsp27. A, p90Rsk phosphorylates Hsp27 in vitro. Purified Hsp27 (1 μg) was incubated with the indicated concentrations of active p90Rsk in kinase buffer in presence of 32P-γ-ATP for 30 min at 30°C. Kinase reactions were terminated using Laemmli buffer and resolved in 10% SDS-PAGE. Gels were stained with Coomassie blue, and then dried and exposed for autoradiography (left). Purified Hsp27 (1 μg) was incubated with 250 μg of purified p90Rsk in the presence or absence of the indicated concentrations of Ro31-8220. Kinase reactions were terminated using Laemmli buffer and resolved in 10% SDS-PAGE. Gels were stained with Coomassie blue, dried, and exposed for autoradiography (right).

B, p90Rsk phosphorylates Hsp27 in vivo. PC-3 cells were transfected with p90Rsk wild-type Flag or DN-Flag cDNA and empty vector for 48 h, then serum-starved overnight and stimulated with IGF-I for 10 min. Proteins were submitted to Western blot using p-Hsp27/T-Hsp27 and p-p90Rsk/T-p90Rsk (left). Right, PC-3 cells were transfected with 20 nmol/L of siRNA p90Rsk2 for 48 h, then serum-starved overnight and stimulated with IGF-I for 10 min. Proteins were submitted to Western blot using p-Hsp27/T-Hsp27 and p-p90Rsk/T-p90Rsk.

C, Hsp27 is associated with p90Rsk. Total proteins (750 μg) were immunoprecipitated with 2 μg of Hsp27, p90Rsk, or control IgG overnight. Immunoprecipitated material was submitted to Western blot using Hsp27 and p90Rsk antibodies (left). p90Rsk wild-type or DN cDNA were transfected into PC-3 cells; 72 h later proteins were extracted and 750 μg of total proteins were pulled down with Flag-Agarose and precipitated materials were recovered by centrifugation. Western blot was performed using T-Hsp27, p-p90Rsk and T-p90Rsk as control loading (right). D, p90Rsk co-localizes with Hsp27 in PC-3 cells. PC-3 cells were fixed with paraformaldehyde and double-stained with Hsp27 (1:500) and p90Rsk (1:100) antibodies (left). p90Rsk and Hsp27 stained the same subsets of the cells in NHT prostate cancer tumors. 3M NHT tumors were stained back-to-back with Hsp27 and p90Rsk, showing that both proteins share the same subset of the cells (right).
which was amplified in the presence of IGF-I stimulation compared with empty vector. However, p90Rsk dominant negative/kinase dead overexpression decreases endogenous Hsp27 and p90Rsk phosphorylation in the absence or presence of IGF-I stimulation (Fig. 4B, left). To further support these results, p90Rsk siRNA decreased p-Hsp27 levels that directly correlated with p90Rsk knockdown (Fig. 4B, right). Interestingly, p90Rsk interacts with Hsp27 (Fig. 4C, left), and this interaction is dependent on p90Rsk activation because the DN of p90Rsk abolished p90Rsk/Hsp27 interaction (Fig. 4C, right). Hsp27 and p90Rsk colocalize in the cytoplasm in PC-3 cells (Fig. 4D, left). Moreover, immunohistochemical analysis of human prostate cancer specimens indicates that Hsp27 and p90Rsk are present in the same subset of the cells after androgen ablation (Fig. 4D, right). Collectively, these results indicate that p90Rsk directly interacts with and phosphorylates Hsp27 in vitro and in vivo, and that this interaction is p90Rsk-dependent.

**Hsp27 stimulates Bad binding to 14-3-3.** Akt and Erk activation leads to phosphorylation of Bad at Ser\(^{112}\) and Ser\(^{136}\), which increases 14-3-3 binding and sequesters Bad in the cytoplasm to suppress its proapoptotic activity (33). Because Hsp27 is a cytoprotective chaperone and is phosphorylated by p90Rsk, and Hsp27 knockdown increases apoptosis rates, we hypothesized that Hsp27 levels may modulate cell survival via the modulation of Bad phosphorylation and formation of the Bad/14-3-3 complex. We first characterized the effects of Hsp27 on the levels of IGF-IR downstream targets after IGF-I stimulation and found that Hsp27 knockdown abolished IGF-I–induced phosphorylation of Hsp27, Akt, Erk, and Bad on Ser\(^{112}\) and Ser\(^{136}\) without affecting their total expression levels (Fig. 5A).

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**Figure 5.** Hsp27 phosphorylation and expression levels correlate with targets of MAPK and Akt pathways, including Bad/14-3-3 complex formation. A, Hsp27 knockdown decreases the levels of downstream targets of MAPK and Akt pathways. PC-3 cells were treated with 10 nmol/L of Scr or siRNA Hsp27 twice and 48 h after transfection, cells were serum-starved overnight and then stimulated with IGF-I for 10 min. Proteins were extracted and used either for direct Western blot using T-Hsp27/p-Hsp27, T-Akt/p-Akt, T-Erk/p-Erk, T-pRsk/p-p90Rsk, T-Bad/p-Bad\(^{112-136}\), or Bad immunoprecipitation followed by Western blot for 14-3-3. B, Hsp27 overexpression increases the levels of downstream targets of MAPK and Akt pathways. PC-3 cells overexpressing Hsp27 wild-type or empty vector were serum-starved overnight and stimulated with IGF-I for 10 min. Proteins were extracted and used either for direct Western blot using T-Hsp27/p-Hsp27, T-Akt/p-Akt, T-Erk/p-Erk, T-pRsk/p-p90Rsk, T-Bad/p-Bad\(^{112-136}\), or Bad immunoprecipitation followed by Western blot for 14-3-3. C, Hsp27 phosphorylated mutants suppress downstream targets of MAPK and Akt pathways. PC-3 cells overexpressing Hsp27 triple mutant or empty vector were serum-starved overnight and stimulated with IGF-I for 10 min. Proteins were extracted and used either for Western blot using T-Hsp27/p-Hsp27, T-Akt/p-Akt, T-Erk/p-Erk, T-pRsk/p-p90Rsk, T-Bad/p-Bad\(^{112-136}\), or Bad immunoprecipitation followed by Western blot for 14-3-3.
contrast, Hsp27 wild-type overexpression enhances the phosphorylation of Akt, Erk, and p90Rsk compared with empty vector (Fig. 5B). An Hsp27 triple mutant (Ser\(^{15}\), Ser\(^{78}\), and Ser\(^{82}\) were mutated with alanine) also decreased the levels of phosphorylated Akt, Erk, and p90Rsk, consistent with its dominant-negative effect and loss of Hsp27 function (Fig. 5C). Next, we investigated the effect of Hsp27 on Bad phosphorylation as a downstream effector of Akt and MAPK pathways. Overexpression of Hsp27 wild-type increases the phosphorylation of Bad and Bad/14-3-3 complex formation, whereas Hsp27 downregulation at expression (siRNA) or phosphorylation (triple mutant) levels decreased the levels of Bad phosphorylation on Ser\(^{112}\) and Ser\(^{136}\) and Bad/14-3-3 complex, even in the presence of IGF-I. We also found that silencing Hsp27 using OGX-427 (data not shown) or siRNA in PC-3 cells treated with or without IGF-I increased the sub-G\(_0\) fraction (Fig. 6A) and caspase-3 activity (Fig. 6B). These results show that Hsp27 silencing increases apoptotic rates and this effect was not reversed by IGF-I. Collectively, these results indicate that the levels of total and p-Hsp27 are important determinants of Bad/14-3-3 complex stability and represent one mechanism regulating cancer cell survival.

**Discussion.** Survival proteins and signaling pathways upregulated following androgen ablation that function to inhibit cell death are of special importance to treatment resistance and the progression of CRPC. Increased activity in the IGF-IR signaling pathway and altered expression of IGF-I axis components are strongly implicated in prostate carcinogenesis and CRPC progression (30, 34), and contributes to nearly 50% of cell growth and proliferation in some models (35). Castrate-resistant progression of human prostate cancer xenografts is associated with increased expression of IGF-IR and IGF-I signaling pathways (30, 36). In prostate cancer and other cancer models, IGF-I is mitogenic and antiapoptotic, and IGF-IR blockade inhibits castration-induced prostate cancer xenograft growth (37). The PI3/Akt and Erk signaling pathways are the principal cell survival pathways activated by the IGF-I axis (38), and include the phosphorylation, and subsequently, deactivation of the pro-apoptotic Bad protein (39). Hsp27 is phosphoactivated during cell stress to form oligomers that prevent protein aggregation and/or regulate activity/degradation of certain client proteins (40). Hsp27 phosphorylation is catalyzed by MAPKAP kinase-2, a target of the p38 MAPK (41, 42). Higher levels of Hsp27 are commonly detected in many cancers (18) in which it plays a role in thermotolerance, cytoprotection, steroid hormone response, and molecular chaperoning (43). Hsp27 is also involved in the regulation of Akt (44) and enhances nuclear factor-κB activity by increasing the degradation of IκBα (29). Hsp27 also chaperones and shuttles Stat3 and AR into the nucleus, enhancing the transcription of several Stat3- and AR-regulated survival genes (8). In prostate cancer, stress-induced increases in Hsp27 after hormone therapy inhibit castration-induced cell death, accelerate CRPC progression, and render cells more resistant to chemotherapy (8, 19).

Because Hsp27 is involved in many pathways regulating the response of a cell to stress and therapeutic stimuli, including MAPK and Akt pathways, and that increased Hsp27 and IGF-I activity are both associated with CRPC progression (19, 30), we explored the interactions between IGF-I signaling and Hsp27 phosphoactivation that could promote castrate-resistant disease. Here, we report that both total and p-Hsp27 are highly expressed in and correlates with CRPC in human prostate tissues (Fig. 1A) and LNCaP xenografts postcastration (Fig. 1B). Increased p-Hsp27 levels are accompanied by Akt, Erk, and p90Rsk phosphorylation, all involved in cell survival and proliferation. We previously reported that Hsp27 phosphorylation plays a key role in AR activation and stability (32), that increased Hsp27 levels accelerate castrate-resistant LNCaP xenograft growth, and that

Figure 6. Hsp27 knockdown induces cell apoptosis. A, effect of Hsp27 knockdown on cell cycle population. PC-3 cells were treated with 10 nmol/L of Hsp27 siRNA or Scr control and then incubated ±IGF-I for 48 h. Cell cycle populations were analyzed using propidium iodide staining (left). B, effect of Hsp27 knockdown on caspase-3 activity. PC-3 cells were treated with 10 nmol/L of Hsp27 siRNA or Scr control and then incubated ±IGF-I for 48 h. Total proteins (50 μg) from each condition were lysed in radioimmunoprecipitation assay buffer and monitored for their ability to cleave the fluorogenic substrate Ac-DEVD-AMC. Fluorescence generated by the cleavage was quantified by using a spectrofluorometer.
Hsp27 knockdown using antisense (OGX-427; OncoGenex Technologies) or siRNA delays progression postcastration. Collectively, these data functionally link phosphoactivated Hsp27 to stress-induced prostate cell survival and castrate-resistant progression (8, 32).

Depending on cell context and stimulus, Hsp27 phosphorylation is regulated by several protein kinases involved in cell survival and proliferation, including p38 kinase and Akt (44, 45). PKCa, PKCδ, and cyclic AMP–dependent kinase have also been reported to phosphorylate Hsp27 (46), suggesting that Hsp27 regulates the activity of many varied signaling pathways. We show here for the first time that Hsp27 is directly phosphorylated by p90Rsk kinase, a downstream effector of MAPK pathway, in an IGF-I–dependent manner. Several lines of evidence support a link between Hsp27 phosphorylation and p90Rsk in prostate cancer progression. First, both Hsp27 and p90Rsk are overexpressed in prostate cancer and both enhance AR transcription activity (32, 47). Using in vitro and in vivo assays, we show that p90Rsk directly phosphorylates Hsp27 and that dominant negative p90Rsk inhibits Hsp27 phosphorylation. Immunoprecipitation and immunofluorescence studies indicate that Hsp27 is associated and colocalized with p90Rsk. Moreover, Hsp27 and p90Rsk are present in the same subsets of the cells in prostate cancer tumors after androgen ablation. Collectively, these studies indicate that p90Rsk interacts and phosphorylated Hsp27, a key functionally relevant mechanism linked to CRPC growth and survival.

p-Hsp27 is a downstream effector of several signaling pathways linked to cell survival and CRPC including MAPK/Erk, PI3K/Akt, and p38 kinase. In the LNCaP xenograft model, increased p-Hsp27 levels correlate with increased levels of phosphorylated Erk, p90Rsk, and Akt, suggesting that growth factor signaling pathways like IGF-I and interleukin-6 are activated after castration. Enhanced IGF-I signaling, mediated by increased IGF-IR (30), IGFBP-2 (48), and IGFBP-5 (7) levels, is believed to be a functionally and clinically relevant driver of CRPC progression (49). Interactions between IGF-I signaling and Hsp27 via activation of MAPK/p90Rsk and Hsp27 phosphorylation lead to Bad/14-3-3 complex stabilization, Bad sequestration in the cytoplasm, and inhibition of apoptosis. In summary, Hsp27 is a key interacting component of the IGF-I signaling in the development of CRPC. Hsp27 is directly phosphorylated by p90Rsk to serve as a downstream effector of IGF-I signaling, promoting cell survival pathways by chaperoning and stabilizing Bad/14-3-3 complexes. These data support the development of targeted inhibitors of Hsp27 in CRPC.

Disclosure of Potential Conflicts of Interest

The University of British Columbia has submitted patent applications, listing M.E. Gleave as inventor, on the antisense sequence described in this article. This IP has been licensed to OncoGenex Pharmaceuticals, a Vancouver-based biotechnology company in which M.E. Gleave has founding shares.

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