Down-regulation of Mammalian Sterile 20–Like Kinase 1 by Heat Shock Protein 70 Mediates Cisplatin Resistance in Prostate Cancer Cells

Aixia Ren, Guijun Yan, Bei You, and Jianxin Sun

Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey

Abstract

Mammalian sterile 20–like kinase 1 (Mst1) is an ubiquitously expressed serine/threonine kinase, and its activation results in cell apoptosis. Recent studies suggest that Mst1 may function as a tumor suppressor. Here, we reported that heat shock protein 70 (Hsp70), which is thought to protect cells against cellular stress, has been identified as an Mst1-interacting protein, in a yeast two-hybrid screen of human adult prostate cDNA library with a dominant-negative Mst1 (K59R) as bait. The interaction of Mst1 with Hsp70 was confirmed by coimmunoprecipitation in both cotransfected HEK293 cells and prostate cancer cells. Hsp70 colocalized with Mst1 in the cytoplasm of LNCaP cells. The interaction sites with Mst1 consisted of NH_2-terminal ATPase domain in Hsp70, whereas the inhibitory domain of Mst1 mediates the binding of Hsp70 in Mst1. Overexpression of Hsp70 mediates proteasomal degradation of Mst1 in a Hsp70 interacting protein (CHIP)-dependent manner. Furthermore, the proapoptotic effect of Mst1 was markedly inhibited by overexpression of Hsp70 or CHIP. Most strikingly, in response to the treatment of anticancer drug cisplatin, the induction of Hsp70 expression is higher in the androgen-independent DU145 cells compared with the androgen-dependent LNCaP cells. The higher levels of Hsp70 induction and subsequent Mst1 degradation mediate cisplatin resistance in prostate cancer DU145 cells. Moreover, overexpression of Mst1 sensitizes prostate cancer cells to cisplatin treatment. These findings implicate that Mst1, a downstream target of Hsp70, may be developed as a target for sensitizing hormone-refractory prostate cancers to chemotherapy. [Cancer Res 2008;68(7):2266–74]

Introduction

Mammalian sterile 20–like kinase 1 (Mst1) is a ubiquitously expressed serine/threonine kinase with a similarity to Ste20, an upstream activator of the mitogen-activated protein kinase (MAPK) pathway in budding yeast (1–3). Mst1 contains an NH_2-terminal catalytic domain and an autoinhibitory segment followed by a dimerization domain and a nuclear localization motif at the noncatalytic, COOH-terminal part (3). In addition, Human Mst1 has two caspase cleavage sites suited between the catalytic and regulatory domains, which mediate the cleavage of the auto-inhibitory domain (3, 4). In response to a variety of apoptotic stimuli, Mst1 is cleaved by caspases to 34- to 36-kDa NH_2-terminal constitutive active fragment, and this cleavage markedly increases Mst1 kinase activity and translocates cleaved Mst1 to the nucleus where it phosphorylates histone H2B on Ser^{14}, resulting in chromatin condensation, DNA fragmentation, and, ultimately, cell death by apoptosis (4–6). In addition to caspase cleavage, Mst1 phosphorylation has recently been proposed to contribute to kinase activation (7, 8). Several phosphorylation sites have now been identified in Mst1, namely Thr^{175}, Thr^{177}, Thr^{183}, Thr^{187}, Ser^{327}, and Thr^{387}(7, 9). Of these, Thr^{183} and Thr^{187} seem to be essential for kinase activation (9, 10). Phosphorylation at these sites may be further amplified by dimerization and eventually leads to caspase activation, thereby constituting a powerful amplification loop of apoptotic responses (10).

Recently, the role of Mst1 in cancer cells has begun to be explored. Recent studies indicate that Mst1 and the related protein Mst2 may play an important role in regulation of cell cycle progression as well as in tumor suppression. Based on its proposed function in apoptosis, Mst1 might function as a tumor suppressor in human cancers as has been suggested for its *Drosophila* homologues. Together with Salvador and Warts, a *Drosophila* homologue of Mst1/2, Hippo, has been shown to promote both proper exit from the cell cycle and apoptosis during development (11–13). In addition, Mst1 has been shown to associate with the tumor suppressor RASSF1A, and this interaction resulted in the full activation of Mst1 during Fas-induced apoptosis and enhanced Mst1-mediated apoptosis *in vivo* (10, 14). Recently, the loss of cytoplasmic Mst1 has been shown to be associated with higher tumor grade and poor prognosis in colorectal cancer patients (15), further suggesting a tumor suppressor role for Mst1 in human colorectal cancer. However, the mechanisms underlying the regulation of the Mst1 activation and its role in chemoresistance in cancer cells remains largely unknown.

In this study, we performed a yeast two-hybrid screen of a human prostate library using the dominant-negative form of Mst1(K59R; DNMst1) as bait to identify novel Mst1-associated proteins. Subsequently, we report evidence that that heat shock protein 70 (Hsp70) is a novel mediator of the Mst1 signaling in prostate cancer cells. Hsp70 directly interacts with and augments proteasomal degradation of Mst1 via a Hsp70 interacting protein (CHIP)-dependent mechanism. We also showed that induction of Hsp70 and subsequent degradation of Mst1 mediate cisplatin resistance in prostate cancer cells.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

A. Ren and G. Yan contributed equally to this work.

Requests for reprints: Jianxin Sun, Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry New Jersey-New Jersey Medical School, 185 South Orange Avenue, MGB G-653, Newark, NJ 07103. Phone: 973-972-7048; Fax: 973-972-7489; E-mail: sunj1@umdnj.edu.

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Materials and Methods

Cell culture. Prostate cancer cell lines DU145 and LNCaP were obtained from American Type Culture Collection and grown in RPMI 1640 (VWR) supplemented with 10% fetal bovine serum (Hyclone Laboratories), 20 units/mL penicillin, and 0.02 mg/mL streptomycin at 37°C under 5% CO_2.

Yeast two-hybrid screening. The MATCHMAKER GAL4 yeast two-hybrid system 3 (Clontech Laboratories, Inc.) was performed, and the human prostate MATCHMAKER cDNA library was screened using human dominant-negative Mst1(K59R) as bait as previously described (16). The screening was performed in AH109 yeast cells according to the protocol provided by the Clontech Matchmaker Two-Hybrid System. Positive colonies were subject to multiple rounds of additional selection in the appropriate medium and β-galactosidase (β-gal) filter assays to verify specificity.

Plasmid constructs. Plasmids bearing full-length Mst1 and Mst1 mutants were generated by PCR and subcloned into pCS2-6XMT vector. Hsp70 and Hsp70 mutants expression vectors were generated by PCR and subcloned into pFLAG-CMV2 vector (Sigma). To generate bacterially expressed Hsp70 and Mst1, the full-length Hsp70 and Mst1 cDNAs were cloned into pET-28a (Novagen) and pGEX-4T (Novagen) expression vectors, respectively. All recombinant plasmids were transformed into *Escherichia.-coli* BL21. The expression of recombinant proteins was induced at 30°C with 1 mmol/L isopropyl-β-D-thiogalactopyranoside for 4 h. Recombinant proteins were purified by Ni^{2+}-nitrilotriacetic acid–agarose or glutathione Sepharose 4B Beads.

Construction of adenoviruses. Adenoviruses harboring Mst1, Hsp70, and CHIP were made using AdMax (Microbix). Briefly, pBHGloxE1,3Cre, including the estrone adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest into Ad293 cells using Fugene 6 (Roche). Adenovirus bearing LacZ was obtained from Clontech. The viruses were propagated on Ad293 cells and purified using $CsCl_2$ banding followed by dialysis against 20 mmol/L TBS with 2% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the instructions of the manufacturer. Adenovirus harboring Mst1-specific siRNA (target sequence, 5′-AAGTGGACCAGGAC-GATGAAG-3′) or Hsp70 siRNA (target sequence, 5′-GAAGGACGAGTTT-GAGCACAA-3′) was constructed using p*Silencer* adeno 1.0-CMV System (Ambion).

Glutathione *S*-transferase pull-down. Purified recombinant His-tagged Hsp72 and glutathione *S*-transferase (GST)-Mst1 were incubated together for 2 h at 4°C in solution containing 150 mmol/L NaCl and 50 mmol/L Tris-HCl (pH 7.5). The beads was extensively washed with 50 mmol/L Tris-HCl (pH 7.5), and bound proteins were eluted from the beads and analyzed by SDS-PAGE.

Coimmunoprecipitation of Hsp70 and Mst1 in HEK293 cells and prostate cancer cells. HEK293T were grown in DMEM (VWR) plus 10% (v/v) fetal bovine serum. Cells were transiently transfected with Mst1 and Hsp70 cDNAs using FuGENE 6, as described by the manufacture (Roche). Coimmunoprecipitation of Mst1 and Hsp70 was performed essentially as described (16). The following antibodies were used for detection and immunoprecipitation: rabbit polyclonal c-Myc (Invitrogen), mouse monoclonal FLAG M2 (Sigma), mouse monoclonal Hsp70 (Stressgene), and rabbit polyclonal Mst1 (Abgent). Secondary antibodies were peroxidase-conjugated donkey anti-rabbit or anti-mouse (Jackson ImmunoResearch). Detection of the peroxidase was performed with enhanced chemiluminescence (Amersham) reagents.

Confocal immunofluorescent staining. The intracellular colocalization of Mst1 with Hsp70 was examined using an immunofluorescent staining procedure as described previously (16). LNCaP cells cultured on chamber slides were washed twice with PBS, fixed in 3.8% formaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min. Cells were washed

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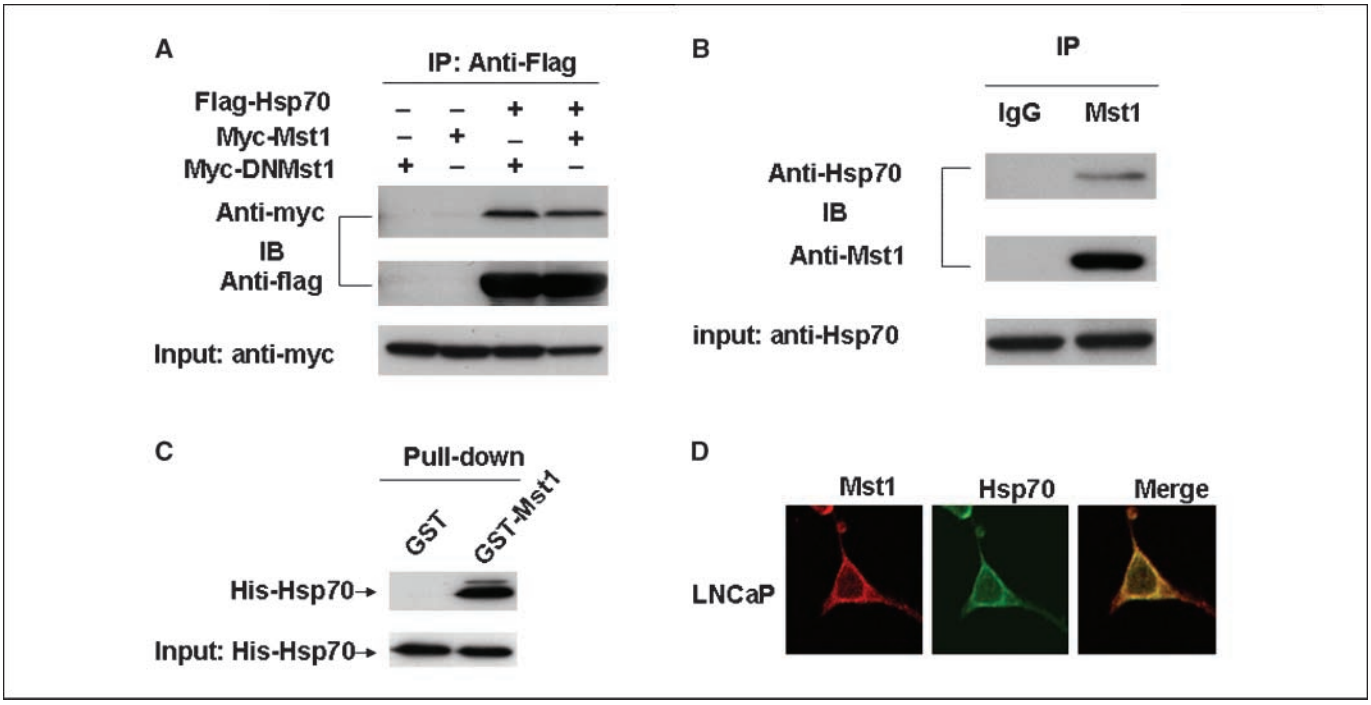

Figure 1. Physical interaction between Mst1 and Hsp70 by immunoprecipitation (*IP*) analysis. *A,* expression vector in combination of either pMT2-Myc-Mst1, pMT2-MycDNMst1, or pFLAG-Hsp70 were cotransfected into HEK293 cells. Extracted proteins were precipitated by anti-Flag antibody and then separated by 10% SDS-PAGE. The transferred membrane was immunoblotted with either horseradish peroxidase (HRP)-conjugated anti-Myc or HRP-conjugated anti-Flag antibody. *B,* cell lysates obtained from prostate cancer LNCaP cells were immunoprecipitated with anti-Mst1 antibody and then separated by 10% SDS-PAGE. Transferred membrane was immunoblotted with either anti-Mst1 or Hsp70 antibody. *C,* recombinant Hsp70 incubated with either GST or GST-Mst1, and proteins were precipitated with glutathione-Sepharose 4B beads. The precipitated proteins were separated through SDS-PAGE, and Western blot analysis was done with anti-Hsp70 monoclonal antibody. *D,* fixed LNCaP cells were stained with anti-Hsp70 monoclonal antibody and rabbit polyclonal anti-Mst1 antibody and processed for confocal imaging. The merged image shows clear colocalization of these two proteins in cytoplasm.

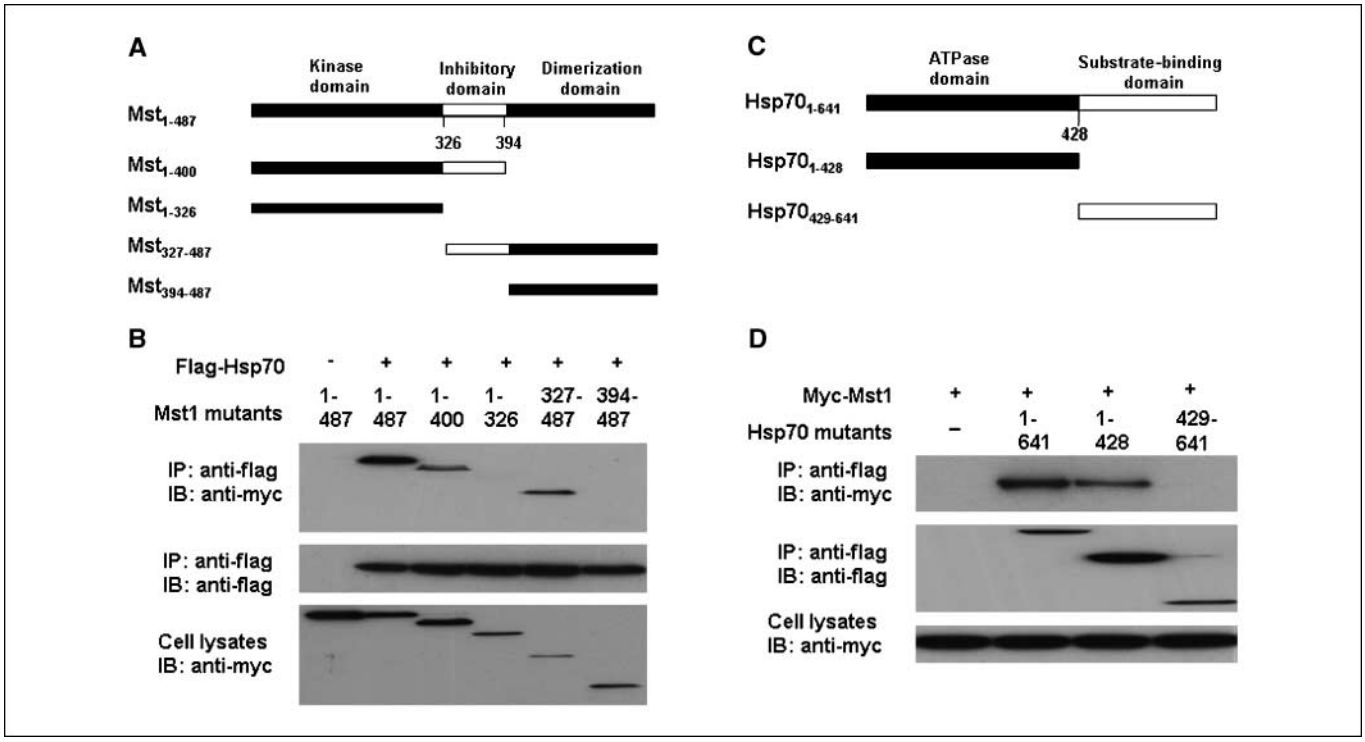

Figure 2. Identification of Mst1 and Hsp70 interaction sites. *A,* schematic representation of Mst1 deletion mutants. *B,* flag-Hsp70 expression vector in combination of either empty vector or expression vectors of Myc-Mst1 mutants were cotransfected into HEK293 cells. Extracted proteins were precipitated by anti-Myc antibody and then separated by 12% SDS-PAGE. The transferred membrane was immunoblotted with either HRP-conjugated anti-FLAG or HRP-conjugated anti-Myc antibody. Lysates were also immunoblotted with anti-Myc antibody to show the expression levels of Myc-Mst1 mutants in HEK293 cells. *C,* schematic representation of Hsp70 deletion mutants. *D,* Myc-Mst1 expression vector in combination of either empty vector or expression vectors of Flag-Hsp70 mutants were cotransfected into HEK293 cells. Extracted proteins were precipitated by anti-Flag antibody and then separated by 12% SDS-PAGE. The transferred membrane was immunoblotted with either HRP-conjugated anti-FLAG or HRP-conjugated anti-Myc antibody. Lysates were also immunoblotted with anti-Myc antibody to show the expression.

twice with PBS, blocked with 2% bovine serum albumin for 1 h, and subsequently incubated with Mst1 polyclonal antibody and Hsp70 monoclonal antibody at 4°C overnight. Cells were washed five times with PBS and incubated with appropriate fluorescent-labeled secondary antibodies at RT for 1 h. Images were visualized using an Olympus IX70 epifluorescence microscope.

In-gel kinase assay. 293T cells were transiently transfected with Myc-DNMst1, Myc-Mst1, and Flag-Hsp70 cDNAs. Forty-eight hours after transfection, cells were lysed in the buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L $MgCl_2$, and 0.5% Triton X-100. Cell lysates were subjected to centrifugation at 12,000 × *g* for 10 min at 4°C. The resulting supernatants were immunoprecipitated with anti-Myc for 2 h at 4°C. Equal amounts of precipitated Mst1 were incubated for 20 min at 30°C with 5 μg myelin basic protein (MBP) in 25 μL kinase buffer 40 mmol/L HEPES-NaOH (pH 7.4), 20 mmol/L $MgCl_2$, 1 mmol/L DTT, and 1 μCi [γ-^{32}P]ATP. Reactions were terminated by the addition of 2 × SDS sample buffer. For *in vitro* kinase assay, purified Mst1 (Upstate) was incubated with increasing concentration of Hsp70 for 2 h at 4°C and then incubated with 5 μg MBP in 25 μL kinase assay buffer for 20 min at 30°C. Reactions were terminated by the addition of 2 × SDS sample buffer, and then loaded to 12% SDS-PAGE and subjected to autoradiography.

Analysis of DNA fragmentation by ELISA. Histone-associated DNA fragments were quantitated by the Cell Death Detection ELISA (Roche; ref. 17). Briefly, the cytoplasmic fractions were added to the 96-well ELISA plates precoated with the antihistone monoclonal antibody and incubated for 90 min at room temperature. After washing, bound nucleosomes were detected by the addition of anti–DNA-peroxidase monoclonal antibody and reacted for 60 min at room temperature. After the addition of substrate, absorbance was read with an ELISA reader at 405 nm.

Statistical analysis. Statistical analysis was performed with one-way ANOVA and Fisher test if appropriate. A P value of <0.05 was considered to be statistically significant. Data are shown as mean ± SE.

Results

Association of Hsp70 with Mst1. Although Mst1 activation has been implicated in cell apoptosis in response to a wide range of stimuli (18), the mechanism mediating the regulation of Mst1 activation, however, is not completely elucidated. To better define its function and to identify additional binding partners, we used DNMst1 (K59R) as bait in a two-hybrid screen of a cDNA library from human prostate. β-gal–based screening identified several positive clones, and sequencing of cDNA identified one of the positive clones as Hsp70 fragment in-frame to the GAL4 activation domain.

To confirm the yeast two-hybrid results, we examined the interaction between Hsp70 and Mst1 in cells. 293T cells were cotransfected with vectors that expressed Myc-tagged Mst1 and Flag-tagged Hsp70. Immunoprecipitation of Flag-tagged Hsp70 led to coimmunoprecipitation of Myc-tagged Mst1 or Myc-tagged DNMst1 when both proteins were cotransfected (Fig. 1*A*). As a control, the anti-FLAG antibody did not immunoprecipitate Myc-tagged Mst1 or Myc-tagged DNMst1 in the absence of Flag-Hsp70 (Fig. 1*A*). To determine whether there is an interaction of endogenous Hsp70 and Mst1 *in vivo*, we performed immunoprecipitation with anti-Mst1 antibody using lysates obtained from prostate cancer cell line LNCaP. Hsp70 coprecipitated with the

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anti-Mst1 antibody but not with the nonimmune IgG control (Fig. 1*B*). These findings indicate that endogenous levels of Mst1 can interact with Hsp70 in prostate cancer cells.

To show a direct protein interaction between Mst1 and hsp70, *GST Pull-down* was performed using bacterially expressed GST fused to full-length Mst1. Sepharose-conjugated GST-Mst1 or GST alone was incubated with *purified His-Hsp70*, and unbound and bound fractions were analyzed by SDS-PAGE and Western blot. Hsp70 bound to GST-Mst1 but not GST alone, confirming a direct protein interaction (Fig. 1*C*). To determine the intracellular localization of this interaction, we performed immunofluorescence staining in prostate cancer cell line LNCaP. Immunofluorescent microscopy showed that Hsp70 and Mst1 are colocalized in the cytoplasm (Fig. 1*D*).

Interaction domains of Hsp70 and Mst1. To further map the interaction domains of Hsp70 and Mst1, we performed immunoprecipitation in HEK293 cells cotransfected with different deletion mutants of Mst1 and Hsp70. We first investigated the binding domains of Hsp70 in Mst1. Mst1 contains NH_2-terminal kinase domain followed by inhibitory domain and COOH-terminal dimerization domain (Fig. 2*A*; ref. 3). We generated a series of Mst1 deletion mutants subcloned into pCS26MT vector with 6× Myc tag and transfected these mutants into HEK293 cells along with Flag-Hsp70. Lysates from transfected HEK293 cells were immunoprecipitated with anti-Flag antibody and analyzed by Western blot analysis using anti-Flag and anti-Myc antibodies. We found that Flag-Hsp70 bound to Mst1 mutant bearing inhibitory and dimerization domains. Further deletion of Mst1 inhibitory domain totally abolished the interaction of Hsp70 with Mst1 (Fig. 2*B*), suggesting that the inhibitory domain of Mst1 is necessary for interaction with Hsp70.

Hsp70 contains two functional domains, namely, NH_2-terminal ATPase domain and COOH-terminal substrate–binding domain (Fig. 2*C*; ref. 19). To map the Mst1-binding domain in Hsp70, Flag-tagged Mst1 mutants were cotransfected into HEK293 cells with Myc-tagged Hsp70. Lysates from transfected HEK293 cells were immunoprecipitated with anti-Flag antibody and analyzed by Western blotting analysis using anti-Flag and anti–Myc-antibodies. As shown in Fig. 2*D*, Mst1 interacted only with Hsp70 NH_2-terminal ATPase domain but not with COOH-terminal substrate–binding domain. Together, these results further suggest that Hsp70 NH_2-terminal ATPase domain mediates its interaction with Mst1.

Hsp70 mediates degradation of Mst1. To determine whether the interaction of Hsp70 with Mst1 has functional consequences in terms of affecting Mst1 activity, we cotransfected Mst1 and Hsp70 into HEK293 cells and determined Mst1 expression and kinase activity. Transfection of Hsp70 markedly decreased both phosphorylation of substrate MBP and autophosphorylation of Mst1 by ~60%, as determined by an *in vitro* kinase assay, using MBP as a substrate (Fig. 3*A*). In addition, transfection of Hsp70 also substantially attenuated Mst1 expression by ~50% (Fig. 3*A*). Furthermore, to determine whether Hsp70 directly targets Mst1, the effect of Hsp70 on Mst1 activity, *in vitro*, was examined. In contrast, in an *in vitro* kinase assay system, incubation of recombinant bioactive Mst1 with increased concentration of recombinant Hsp70 had no significant effects on Mst1 kinase activity (Fig. 3*B*). In addition, Hsp70 was not phosphorylated by Mst1, suggesting that Hsp70 is not a substrate of Mst1 (data not shown). Moreover, transfection of Hsp70 markedly down-regulated Mst1 expression in a dose-dependent manner in HEK293 cells (Fig. 3*C*). To determine whether Mst1 could serve as a substrate of the proteasomal degradation machinery, which is linked to the

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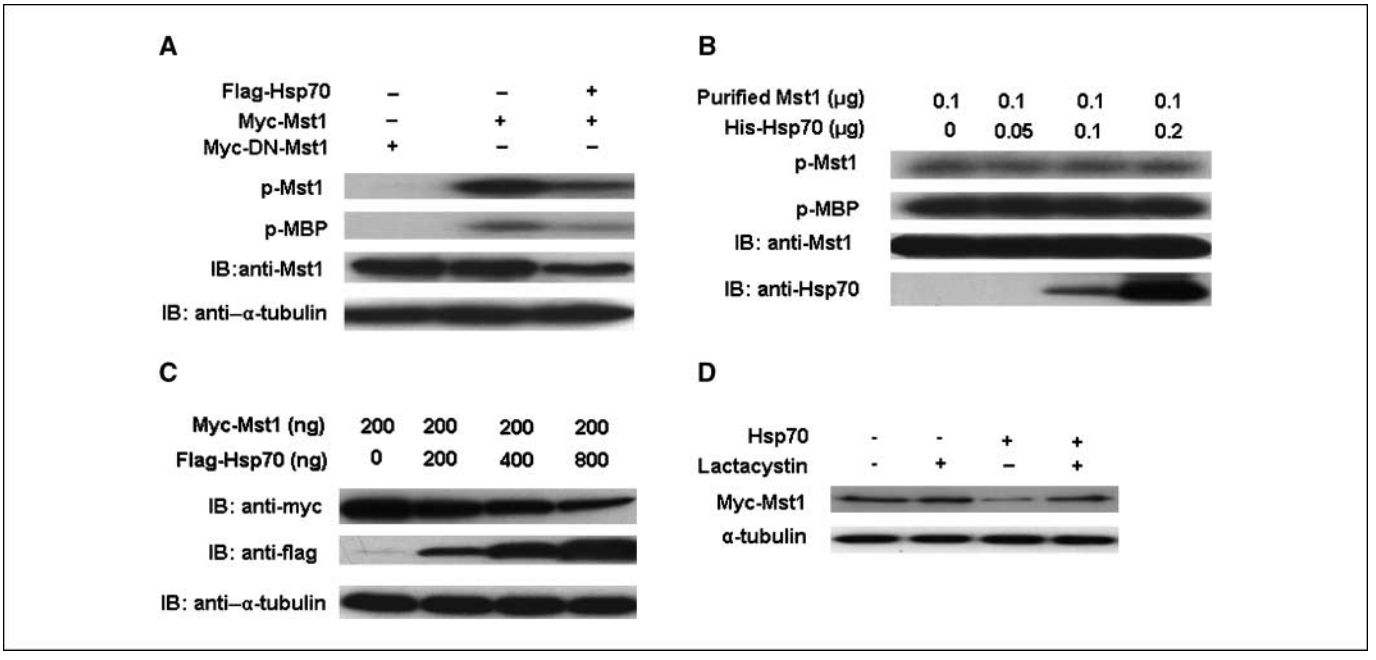

Figure 3. Down-regulation of Mst1 expression by Hsp70. *A,* cell lysates from 293T cells transfected with either expression vectors of Mst1, DN-Mst1, or Mst1+Hsp70 were immunoprecipitated with anti-Myc antibody and inmmunoprecipitates were subjected to in gel kinase assay using MBP as a substrate. *B,* recombinant Mst1 was preincubated with increasing amounts of Hsp70 and then subjected to *in vitro* kinase assay. *C,* Myc-Mst1 expression vector in combination of either empty vector or increasing concentration of Flag-Hsp70 plasmids were cotransfected into HEK293 cells. The expression of Mst1 and Hsp70 were detected by Western blotting analysis. *D,* 293 cells were transfected with Myc-Mst1 expression plasmid together with either empty vector or Hsp70 expression vector. At 48 h after transfection, cells were left untreated or were treated with 10 μmol/L lactacystin for 3 h. Cells were lysed and processed for immunoblotting with anti-Myc antibody. As a control for protein loading, immunoblots (*IB*) were probed in parallel with an antitubulin antibody.

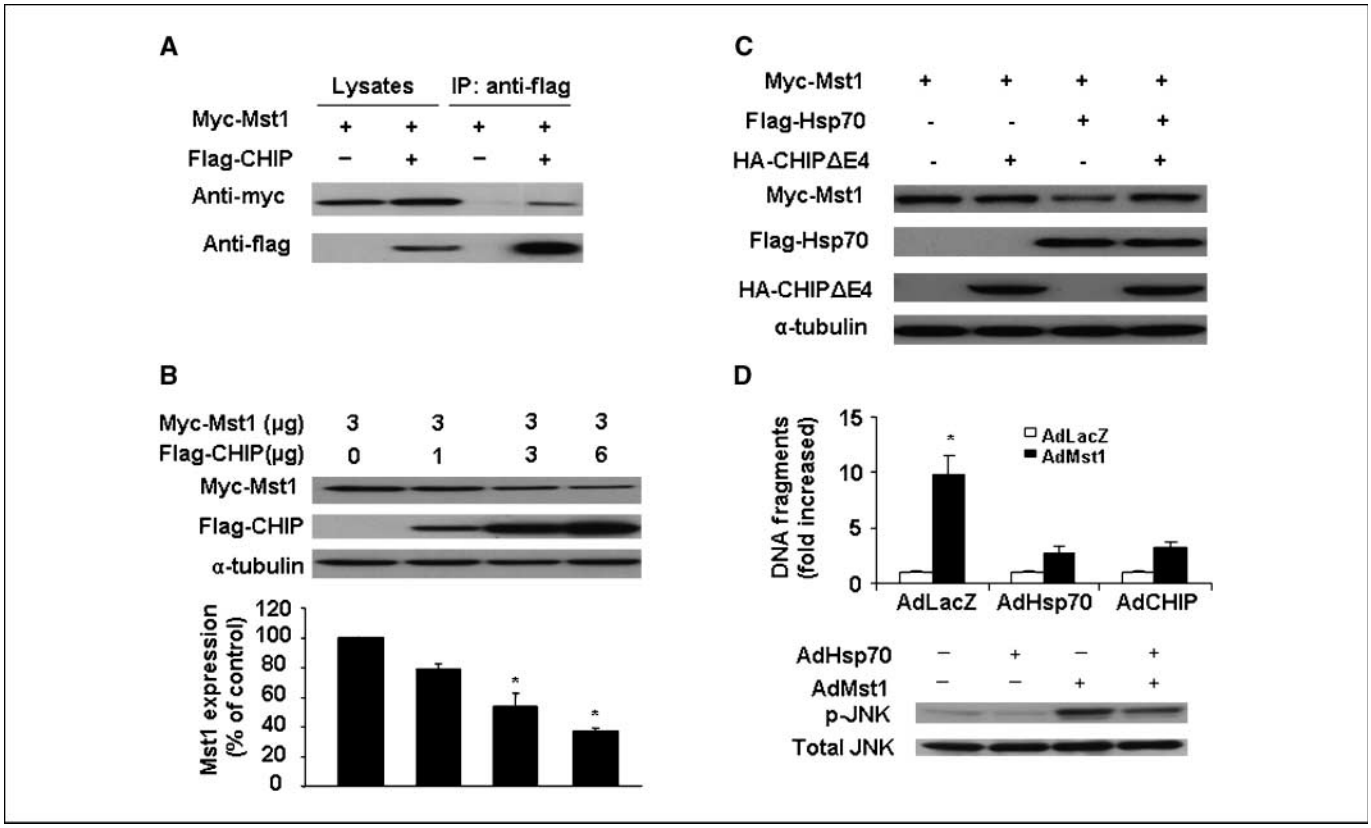

Figure 4. CHIP is required for Hsp70-mediated Mst1 degradation. *A,* expression vector in combination of either pMT2-Myc-Mst1 or pFlag-CHIP were cotransfected into HEK293 cells. Extracted proteins were precipitated by anti-Flag antibody and then separated by 10% SDS-PAGE. The transferred membrane was immunoblotted with either HRP-conjugated anti-Myc or HRP-conjugated anti-Flag antibody. *B,* Myc-Mst1 expression vector in combination of either empty vector or increasing concentration of Flag-CHIP plasmids were cotransfected into HEK293 cells. The expression of Mst1 and CHIP were detected by Western blotting analysis and quantitated by densitometry. *, $P < 0.05$ versus Flag-CHIP at 0 μg. *C*, 293 cells were transiently transfected with Myc-Mst1 expression vector either alone or together with a vector for Hsp70 in the absence or presence of an expression vector for an HA-tagged CHIP mutant lacking ubiquitin ligase activity (*HA-CHIPΔE4*). At 48 h after transfection cells were lysed and processed for immunoblotting with anti-Myc, anti-Flag, or anti-HA antibodies. *D,* LNCaP cells were infected with AdMst1 at a MOI of 30 together with either AdLacZ or AdHsp70 or AdCHIP at MOI of 50. Forty-eight hours after transduction, cell apoptosis was quantitated by the Cell Death Detection ELISA (Roche). JNK and its phosphorylation were determined by Western blotting analysis. The data are representative of four independent experiments. *, $P < 0.05$ versus AdHsp70 or AdCHIP.

heat shock protein network (20), the effect of Hsp70 ovexpression on Mst1 down-regulation was examined in the presence and absence of the proteasome inhibitor lactacystin. As shown in Fig. 3*D*, lactacystin treatment substantially suppresses the disappearance of Mst1 in Hsp70-transfected cells, thereby suggesting that the 26 S proteasome might be responsible for the down-regulation of Mst1 in response to Hsp70 overexpression.

CHIP is required for Hsp70-mediated degradation of Mst1. Previous studies suggest that the cochaperone CHIP, an E3 ubiquitin ligase of the U-box protein family, is involved in the Hsp70-mediated proteasomal degradation (21, 22). To investigate whether CHIP is required for Hsp70-mediated degradation of Mst1, we first examined by coimmunoprecipitation assay whether Mst1 associates with CHIP in cotransfected HEK293 cells. As shown in Fig. 4*A*, immunoprecipitation of Flag-tagged CHIP led to coimmunoprecipitation of Myc-tagged Mst1 when both proteins were cotransfected. As a control, the anti-Flag antibody did not immunoprecipitate Myc-tagged Mst1 in the absence of Flag-CHIP. These studies showed that CHIP is present in immunoprecipitates of Mst1. Furthermore, the expression of Mst1 was markedly attenuated by cotransfection of CHIP in HEK293 cells (Fig. 4*B*). To further confirm a role for CHIP in Hsp70-mediated degradation of Mst1, we used a mutant form of CHIP lacking ubiquitin ligase activity (Myc-CHIPE4; ref. 20). As expected from the above data, expression of Hsp70 greatly reduced the steady-state levels of Mst1 in transfected cells. However, inhibition of endogenous CHIP activity by overexpression of its U-box mutant almost completely antagonizes the effects of Hsp70 on Mst1 (Fig. 4*C*), suggesting that Hsp70 induces Mst1 degradation in a CHIP-dependent manner. Because Mst1 has been characterized to induce cell apoptosis (18), we investigated whether Hsp70 and CHIP can affect apoptosis via inhibitory effects on Mst1 in prostate cancer cells. Indeed, transduction of LNCaP cells with AdMst1 [multiplicity of infection (MOI), 30] significantly induced cell apoptosis, as determined by the extent of DNA fragmentation. The proapoptotic effect of Mst1, however, was markedly attenuated by overexpression of either Hsp70 or cochaperone CHIP. In addition, Mst1-induced phosphorylation of c-Jun-NH_2-kinase (JNK) was also inhibited by overexpression of Hsp70 in LNCaP cells (Fig. 4*D*). Taken together, these findings suggest that the interaction of Hsp70 with Mst1 may be functionally important in terms of regulating Mst1-mediated cell apoptosis.

The Hsp70-Mst1 axis mediates cisplatin resistance in prostate cancer cells. To substantiate the functional significance of the Hsp70-Mst1 interaction *in vivo*, we investigated the role of the Hsp70-Mst1 interaction in cancer cell apoptosis and survival.

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To this end, two types of prostate cancer cell lines, including DU145 and LNCaP cells, were used. Cells were treated with increasing concentration of anticancer drug cisplatin, and the expression of Hsp70 and Mst1 was then detected by Western blotting analysis. As shown in Fig. 5*A* and *B*, treatment of human prostate cancer cells with cisplatin markedly increased the expression of Hsp70. Accordingly, the expression of Mst1 was substantially inhibited. The inhibition of Mst1 expression was not attributable to the caspase-mediated cleavage, as observed in previous studies (4–6), because the 34-kDa NH_2-terminal fragment was not detected in our experiments (data not shown). Most strikingly, the pattern of Hsp70 induction by cisplatin is different in androgen-independent DU145 cells versus androgen-dependent LNCaP cells. Three micromoles per liter of cisplatin markedly induced Hsp70 expression by 2-fold and inhibited Mst1 expression by ~50% in the androgen-independent DU145 cells (Fig. 5*A*). In contrast, in androgen-dependent LNCaP cells, cisplatin, at the same concentration, had no effect on both Hsp70 and Mst1 expression (Fig. 5*B*). At the concentration of 24 μmol/L, cisplatin robustly induced Hsp70 expression by ~10-fold in DU145 cells, compared with 2.5-fold induction in LNCaP cells, although the expression of Mst1 was markedly inhibited in both cell lines (Fig. 5*A* and *B*). Given that Hsp70 plays an important role in mediating chemoresistance in a variety of cancer cells, we hypothesized that the androgen-independent DU145 cells may be more resistant to anticancer drugs, such as cisplatin, in addition to their resistance to androgen ablation treatment. To test this hypothesis, we compared the apoptosis induced by cisplatin between DU145 and LNCaP cells. As shown in Fig. 5*C*, the two cell lines displayed different cisplatin sensitivities. LNCaP cells were more sensitive to the apoptosis induced by cisplatin compared with DU145 cells.

To test whether Mst1 plays a similar role as Hsp70 in mediating chemosensitivity in prostate cancer cells, the expression of Mst1 was knockdowned by adenovirus-mediated overexpression of Mst1-specific siRNA in LNCaP cells. As shown in Fig. 5*D*, knockdown of Mst1 expression by siRNA significantly decreased

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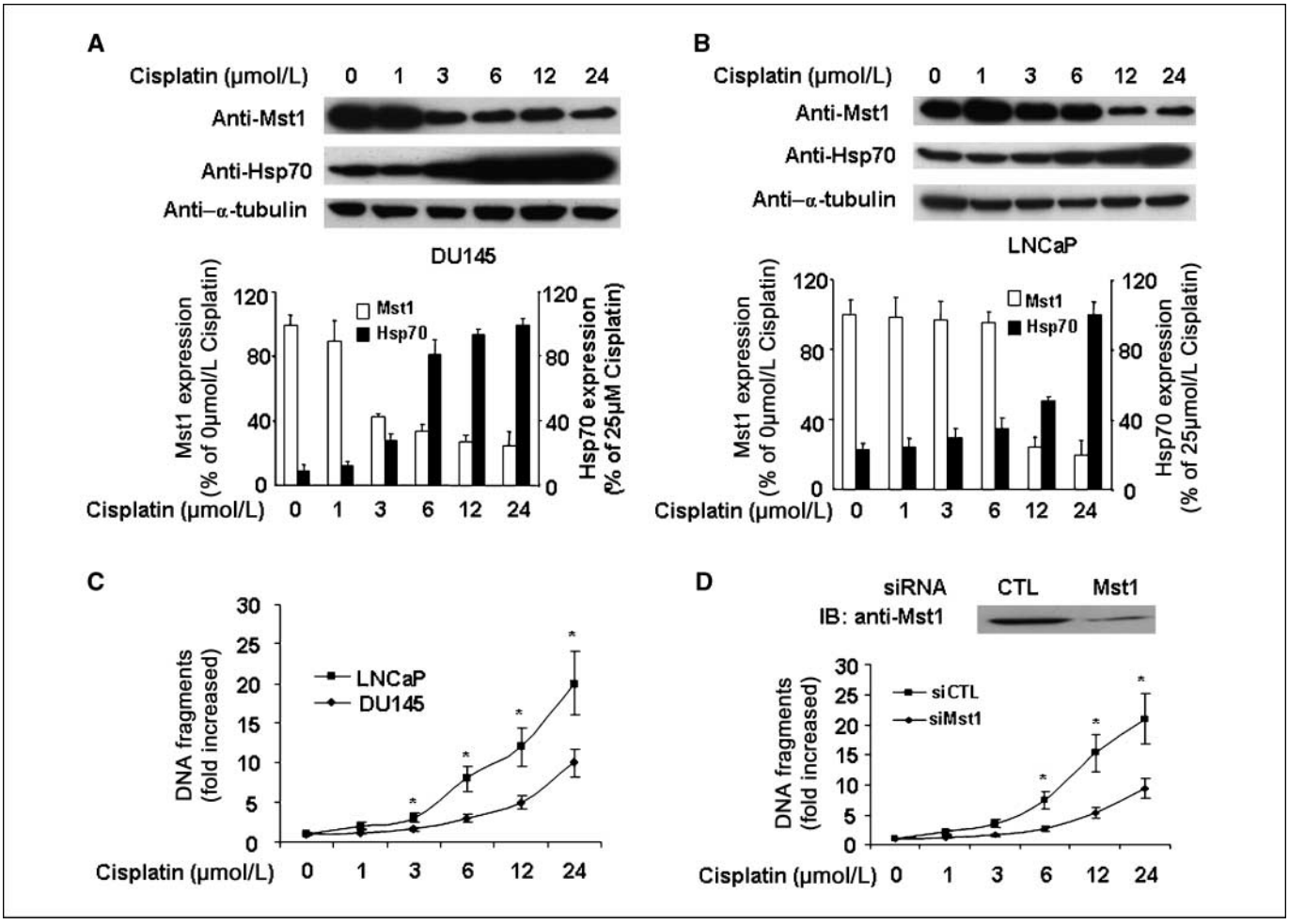

Figure 5. Induction of Hsp70 expression and Mst1 degradation by anticancer drug cisplatin in prostate cancer cells. *A,* prostate cancer DU145 cells were treated with increasing concentration of cisplatin as indicated. Twenty-four hours after treatment, cells were lysed and processed for immunoblotting with anti-Mst1, anti-Hsp70, or anti–α-tubulin antibodies. The expression levels of Mst1 and Hsp70 were quantitated by densitometry. *B,* prostate cancer LNCaP cells were treated with increasing concentration of cisplatin as indicated. Twenty-four hours after treatment, cells were lysed and processed for immunoblotting with anti-Mst1, anti-Hsp70, or antitubulin antibodies. The expression levels of Mst1 and Hsp70 were quantitated by densitometry. *C,* prostate cancer cells were treated with increasing concentration of cisplatin. Forty-eight hours after treatment, after transduction, cell apoptosis was quantitated by the Cell Death Detection ELISA (Roche). The data are representative of four independent experiments. *, $P < 0.05$ versus DU145. *D,* LNCaP cells were transduced with andenovirus harboring Mst1-specific siRNA (*siMst1*) or control siRNA (*siCTL*) at MOI of 50. Seventy-two hours after transduction, cells were lysed and subjected to Western blot analysis of Mst1 expression. In addition, cells were treated with increasing concentration of cisplatin for 48 h. Cell apoptosis was quantitated by the Cell Death Detection ELISA (Roche). The data are representative of four independent experiments. *, $P < 0.05$ versus siMst1.

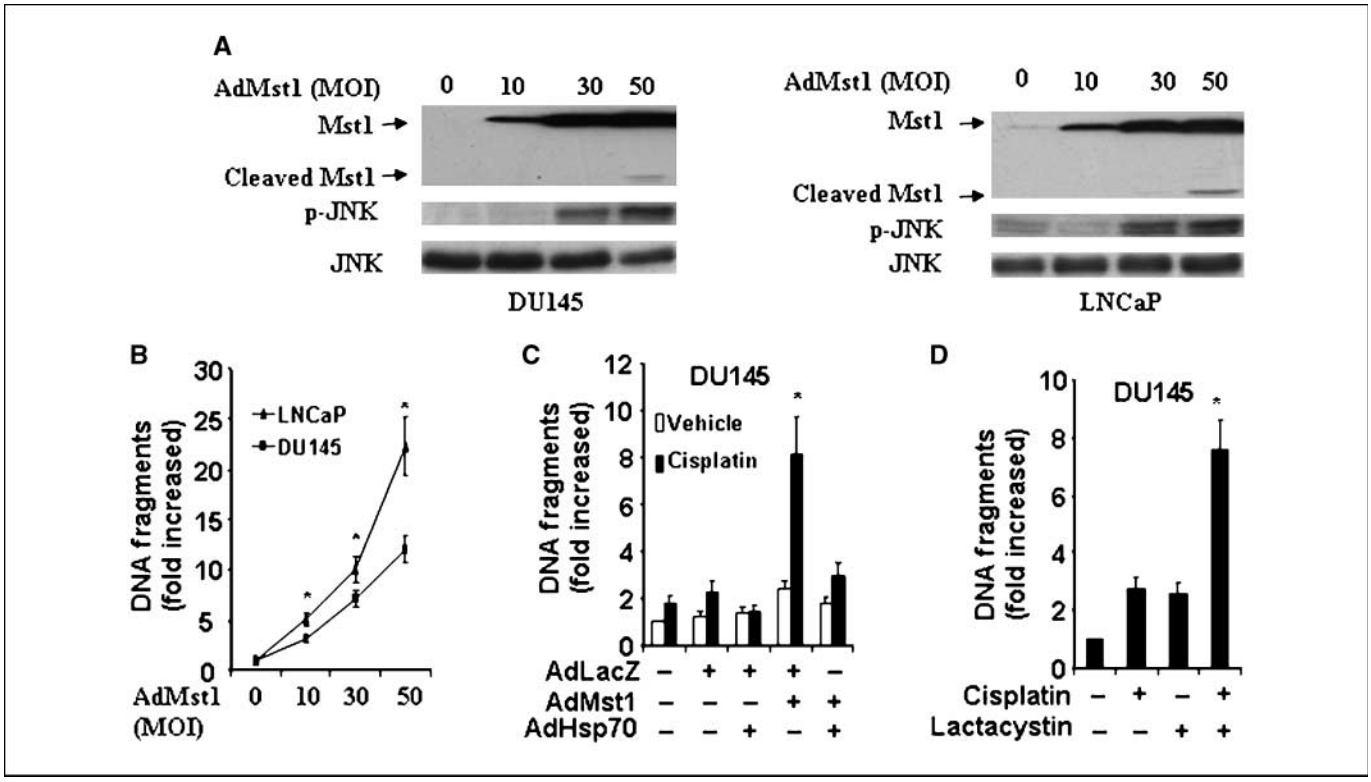

Figure 6. Overexpression of Mst1 sensitizes cisplatin-induced apoptosis in prostate cancer cells. *A,* prostate cancer cells were transduced with increasing MOI of AdMst1. Forty-eight hours after transduction, expression of Mst1 and cleaved form, JNK, and its phosphorylation were detected by Western blotting analysis. *B,* prostate cancer cells were transduced with increasing MOI of AdMst1. Forty-eight hours after transduction, cell apoptosis was quantitated by the Cell Death Detection ELISA (Roche). The data are representative of four independent experiments. *, $P < 0.05$ versus DU145. *C,* DU145 cells were transduced in combination of either AdLacZ, AdMst1, or AdHsp70 at a total MOI of 50 for 24 h, followed by 6 μmol/L cisplatin treatment for additional 48 h. AdMst1 and AdHsp70 were used at MOI of 5 and 45, respectively. Cell apoptosis was quantitated by the Cell Death Detection ELISA (Roche). The data are representative of four independent experiments. *, $P < 0.05$ versus AdLacZ or AdMst1/AdHsp70. *D,* DU145 cells were treated with 6 μmol/L cisplatin, 10 μmol/L lactacystin, or in combination for 48 h, and cell apoptosis was quantitated by the Cell Death Detection ELISA (Roche). The data are representative of four independent experiments.

the sensitivity of LNCaP cells to the cisplatin-induced apoptosis. In contrast, knockdown of Hsp70 by siRNA prevented Mst1 degradation by cisplatin and sensitized cisplatin-induced apoptosis in DU145 cells (Supplementary Fig. S1). Together, these results suggest that the Hsp70-Mst1 axis might be involved in the resistance of human prostate cancer to cisplatin-induced apoptosis.

Overexpression of Mst1 sensitizes cisplatin-induced apoptosis in prostate cancer cells. To further clarify the role of Mst1 in chemosensitivity in prostate cancer cells, we examined the effects of overexpression of Mst1 on cell apoptosis. As shown in Fig. 6*A*, infection with AdMst1 dose-dependently increased expression of both uncleaved and cleaved Mst1 in both DU145 and LNCaP cells. In addition, AdMst1 dose-dependently induced the phosphorylation of JNK (Fig. 6*A*), suggesting that JNK was activated downstream from Mst1. Furthermore, overexpression of Mst1 dose-dependently induced cell apoptosis in both prostate cancer cells, as determined by Cell Death ELISA (Fig. 6*B*). However, the magnitude of the apoptotic effect of Mst1 overexpression in LNCaP cells is higher than that in DU145 cells. Importantly, in combination with cisplatin, AdMst1 synergistically augmented the apoptotic effects of cisplatin in DU145 cells, whereas cotransduction of AdHsp70 substantially inhibited Mst1-induced synergistic effects (Fig. 6*C*). Furthermore, Lactacystin, which prevents degradation of Mst1 by Hsp70 (Fig. 3*D*), also potentiated the cisplatin-induced apoptotic effect in DU145 cells (Fig. 6*D*). Together, these results suggest that the activation of Mst1 may represent an important therapeutic strategy for improving chemotherapeutic efficacy in prostate cancer patients.

Discussion

Hormone-refractory prostate cancers have long been considered a chemoresistant disease. In this study, we found that in response to the treatment of anticancer drugs, such as cisplatin, the induction of Hsp70 expression is higher in the androgen-independent DU145 cells compared with the androgen-dependent LNCaP cells. The higher level of Hsp70 seems to cause a proteasomal degradation of Mst1, a proapoptotic kinase and a potential tumor suppressor, via a CHIP-dependent mechanism. Overexpression of Mst1 sensitized these cells to drug treatment. These findings implicate that Mst1, a downstream target of Hsp70, may be developed as a target for sensitizing hormone-refractory prostate cancers to chemotherapy.

It is well-documented that Hsp70, when induced in the cellular response to cytotoxic stress, prevents cell death triggered by a variety of apoptotic stimuli, including heat shock, tumor necrosis factor (TNF), growth factor withdrawal, oxidative stress, chemotherapeutic agents, ceramide, and ionizing radiation (23). Several different mechanisms have been proposed to account for the cytoprotective activity of Hsp70. Hsp70 has been shown to down-regulate the caspase, JNK, p38 MAPK, and apoptosis signal-regulating kinase signaling cascades (23, 24). Moreover, by

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associating with Apaf-1, Hsp70 blocks Apaf-1/cytochrome *c*–mediated caspase activation (25, 26). Hsp70 also binds to and antagonizes apoptosis-inducing factor, thereby inhibiting caspase-independent apoptosis (27). Recently, accumulating evidence suggests that Mst1 plays a central role in the initiation of cell apoptosis. Indeed, in response to various proapoptotic stimuli and cellular stresses, including TNF-α (28), serum starvation (29), staurosporine (28), anti-Fas monoclonal antibody (30, 31), UV irradiation (28), retinoic acid (28), okadaic acid (32, 33), as well as anticancer drugs (32, 34, 35), Mst1 has been shown to be activated in a wide range of cell lines. The broad nature of the stimuli and the range of the cell systems in which Mst1 is activated suggest that this enzyme may be a common component in the diverse signaling pathways leading to apoptosis. In the present study, our data indicate that Hsp70 may exert cytoprotective effects by directly interacting with and neutralizing Mst1. Given that Mst1 is a MAP4K that contributes to both the JNK and p38 MAPK signaling cascades (7), inhibition of Mst1 by Hsp70 may also be an important mechanism by which Hsp70 inhibits the JNK signaling pathway (34).

Previous studies have indicated that Hsp70 plays essential roles in oncogenesis and in resistance to chemotherapy. Hsp70 overexpression in cancer cells increases their tumorigenicity in rodent models (36, 37). High Hsp70 expression, as observed in most cancer cells, has been associated with metastasis, poor prognosis, and resistance to chemotherapy or radiation therapy (38–41). Furthermore, Hsp70 antisense constructs have been reported to chemosensitize and even kill cancer cell lines in the absence of additional stimuli (42). The cytotoxic effect of Hsp70 down-modulation is particularly strong in transformed cells yet undetectable in normal, nontransformed cell lines or primary cells (42). Thus, Hsp70 seems as an interesting molecular target for sensitizing tumor cell to cancer therapy. However, to date, there are no small molecules currently available to selectively inhibit Hsp70. Thus, the identification of Mst1 as a downstream target of Hsp70 is crucial because Mst1 has been shown to be activated by a variety of chemical and biological agents in a number of cell lines (18). Therefore, it is tempting to speculate that similar agents might be used alone or in combination with other molecules to overcome chemoresistance in cancer cells that overexpress higher levels of Hsp70 protein. This hypothesis is subject to ongoing investigation.

Recently, it has been suggested that Mst1 might function as a tumor suppressor in human cancers as has been suggested for its *Drosophila* homologues. Together with Salvador, the *Drosophila* homologue of hWW45, and Warts, the *Drosophila* homologue of large tumor suppressor (LATS), Hippo, the *Drosophila* homologue of Mst1/2, has been shown to promote apoptosis and proper termination of cell proliferation during development (11, 12, 43). Although mutations in the hWW45 gene have been reported in cancer cell lines (44), its function has not been elucidated in detail. The roles of hWW45 or LATS in resistance to chemotherapy and the relationship between Hsp70, Mst1, and hWW45 or LATS in cancer cells, especially in prostate cancer cells, require further investigation.

In conclusion, the data reported here represent the first demonstration that Mst1 is an intracellular target of Hsp70, and that induction of Hsp70 and subsequent inhibition of Mst1 activation may constitute an important mechanism by which Hsp70 mediates the responses of tumor cells toward chemotherapy. Thus, activation of Mst1 may represent a new approach to enhance the clinical efficacy of prostate cancer chemotherapy.

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