Analysis of Properties of Small Heat Shock Protein Hsp25 in MAPK-activated Protein Kinase 2 (MK2)-deficient Cells

MK2-DEPENDENT INSOLUBILIZATION OF Hsp25 OLIGOMERS CORRELATES WITH SUSCEPTIBILITY TO STRESS*

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Small heat shock proteins (sHsps) exist in dynamic oligomeric complexes and display diverse biological functions ranging from chaperone properties to modulator of apoptosis. So far, the role of stress-dependent phosphorylation of mammalian sHsps for its structure and function has been analyzed by using various phosphorylation site mutants overexpressed in different cell types as well as by non-exclusive inhibitors of the p38 MAPK cascade. Here we investigate the role of phosphorylation of endogenous sHsp in a genetic model lacking the major Hsp25 kinase, the MAP kinase-activated protein kinase MK2. We demonstrate that in MK2-deficient fibroblasts, where no stress-dependent phosphorylation of Hsp25 at Ser86 and no in vitro binding to 14-3-3 was detectable, stress-dependent disaggregation of endogenous Hsp25 complexes is impaired and kinetics of arsinite-dependent, H2O2-dependent, and sublethal heat shock-induced insolubilization of Hsp25 is delayed. Similarly, green fluorescent protein-tagged Hsp25 shows retarded subcellular accumulation into stress granules in MK2-deficient cells after arsinite treatment. Decreased insolubilization of Hsp25 in MK2-deficient cells correlates with increased resistance against arsinite, H2O2, and sublethal heat shock treatment and with decreased apoptosis. In contrast, after severe, lethal heat shock MK2-deficient embryonic fibroblasts cells show fast and complete insolubilization of Hsp25 independent of MK2 and no increased stress resistance. Hence, MK2-dependent formation of insoluble stress granules and irreversible cell damage by oxidative stresses and sublethal heat shock correlate and only upon severe, lethal heat shock MK2-independent processes could determine insolubilization of Hsp25 and are more relevant for cellular stress damage.

sHsps contain the well conserved α-crystallin domain and are present as a family of ten structurally related proteins in humans. While recently of potential genetic relevance for several human diseases (2, 3), sHsps display seemingly diverse cellular functions that still lack the understanding of a unifying molecular mechanisms of action (4). In mammalian cells, most sHsps exist as large oligomeric complexes (5), which are probably stabilized by complex interactions between dimeric building blocks as demonstrated for bacteria and plants (6, 7).

Upon stress, some mammalian sHsps are rapidly phosphorylated via the p38 MAPK/MK2 pathway (8–10). In mouse Hsp25, two phosphorylation sites for MK2, serine (Ser) 15 and Ser86, were identified, whereas three sites exist in human Hsp27, Ser15, Ser78, and Ser82 (10–12). In addition, Thr143 of Hsp27 can be phosphorylated by cGMP-dependent protein kinase (13), and a number of further kinases were described to phosphorylate Hsp25/27, such as MK3 (14), MK5 (15), PKCδ (16), and PKD (17). Phosphorylation of mammalian sHsps is paralleled by disaggregation of their oligomeric complexes leading to dimers or tetramers (18). As functional consequences of phosphorylation and disaggregation, actin stabilizing and stress-protective activity as well as chaperone properties of these proteins were demonstrated to be modulated in different directions. This mainly depends on the experimental setting and so far was always based on overexpression of sHsps and their phosphorylation site mutants (19, 20) or on pharmacological small molecular inhibitors for the upstream kinases p38 MAPKα,β (21, 22), which display non-exclusive specificity (23, 24). For example, non-phosphorylated large oligomers of mouse Hsp25 are described more effective as molecular chaperone (25) and more efficient in protecting cells from tumor necrosis factor α or oxidative stress than the phosphorylated dimers (26), while phosporylation and disaggregation of rat and human Hsp27 was described to be essential for protection against heat shock (27), oxidative stress, and anti-cancer drugs (28).

Here we analyze properties of endogenous Hsp25 in genetically altered cells where a part of the gene for the major Hsp kinase MK2 is deleted leading to the complete loss of MK2 activity (29). In these cells, Hsp25 completely lacks both Ser86 phosphorylation and stress-dependent disaggregation. In addition, insolubilization of Hsp25 is delayed in response to arsinite, H2O2, and sublethal heat shock treatment, the resulting consequences in stress resistance are described.

EXPERIMENTAL PROCEDURES

Materials—The fetal calf serum and penicillin/streptomycin were from PAA (Pasching, Austria). Specific p38 inhibitor

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2 The abbreviations used are: Hsp, heat shock protein; sHsp, small Hsp; GA, glutaraldehyde; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MK, MAPK-activated protein kinase; PK, protein kinase; WT, wild type; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione S-transferase; PBS, phosphate-buffered saline.
SB203580 and its inactive form SB202474 were from Calbiochem-Novabiochem Corp. (La Jolla, CA). The MK2-specific inhibitor was obtained from Dr. A. Mengel, Schering AG (Berlin, Germany).

Rabbit polyclonal antibodies specific for Hsp25 were from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Phospho-Ser82 Hsp27-specific monoclonal antibody, which can also recognize phospho-Ser80 of Hsp25, was a kind gift from Prof. Roy Quinlan (The University of Dundee, Scotland, UK). Phospho-Ser139-specific Hsp25 antibodies were obtained from Stressgen (Victoria, Canada). Antibodies against MK2 and phospho-p38 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-p38α, anti-actin, and anti-IgG secondary antibodies were antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine was obtained from Invitrogen Corp. WST-1 kit was from Roche (Penzberg, Germany). All other chemicals were from Sigma.

Plasmids—EGFP-Hsp27 codes for wild type Hsp27 and was cloned by restriction digestion of pcDNA3-HA-p27-wt (25) with EcoRI and BamHI and ligation into pEGFP-C2 plasmid (Clontech, Palo Alto, CA). pGEX plasmids coding for GST-fused 14-3-3 proteins β, ζ, η, and τ were a kind gift from Dr. Ari Elson (Weizmann Institute of Science) and pGEX-GST-fused 14-3-3γ, ε, and σ were a kind gift from Dr. Michael Yaffe (Massachusetts Institute of Technology).

Cell Culture, Treatment, and Transient Transfection—Primary mouse embryonic fibroblasts (MEFs) from wild type (WT) and MK2-deficient mice were immortalized by transfection with SV40 large T antigen (60). Primary and immortalized MEF cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere 5% CO2 and 95% air. 70–90% confluent cells were pretreated for 30 min with 10 μM SBSB203580 or SB202474 if required and treated with heat shock, H2O2, or sodium arsenite as indicated. Cells were transfected when they reached about 70% confluence. Transfection was carried out on two consequently connected Superose 6HR 30/10 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated with 0.05M K2HPO4/KH2PO4, pH 7.2, 0.15 M NaCl. As a molecular markers, the following combination of proteins were used: tyroglobulin (660 kDa), catalase (230 kDa), bovine serum albumin (67 kDa), and cytochrome (12 kDa). Proteins from fractions were precipitated with trichloroacetic acid-deoxycholate: to 1 volume of protein solution 5% powered skim milk and then incubated with primary antibody (dilution in accordance to manufacture recommendations) overnight at 4 °C. Then membranes were washed three times with TPBS and incubated for 1 h with horseradish peroxidase-conjugated antibody (dilution 1:1000 in blocking solution), washed three times with TPBS, and developed with a chemiluminescence ECL kit using a LAS-3000 (Fuji) imager.

Western Blot Analysis—Equal amounts of protein were boiled for 3 min in 4 × SDS-Laemmli sample buffer (40% glycerol, 4% SDS, 4% β-mercaptoethanol, 0.4 M Tris-HCl at pH 6.7 and 2 mg/ml bromphenol blue) and then separated by 12% SDS-PAGE and transferred onto Hybond ECL nitrocellulose membranes. The membranes were blocked 1 h in 0.01% Tween 20-PBS (TPBS) containing 5% powered skim milk and then incubated with primary antibody (dilution in accordance to manufacture recommendations) overnight at 4 °C. Then membranes were washed three times with TPBS and incubated for 1 h with horseradish peroxidase-conjugated antibody (dilution 1:1000 in blocking solution), washed three times with TPBS, and developed with a chemiluminescence ECL kit using a LAS-3000 (Fuji) imager.

Gel Filtration—Size exclusion liquid chromatography was carried out on two consequently connected Superose 6HR 30/10 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated with 0.05 M K2HPO4/KH2PO4, pH 7.2, 0.15 M NaCl. As a molecular markers, the following combination of proteins were used: tyroglobulin (660 kDa), catalase (230 kDa), bovine serum albumin (67 kDa), and cytochrome (12 kDa). Proteins from fractions were precipitated with trichloroacetic acid-deoxycholate: to 1 volume of protein solution 0.01 volume of 2% deoxycholate was added. After vortex and incubation for 30 min at 4 °C, 0.10 volume of 100% trichloroacetic acid was added. After vortex, samples were incubated overnight at 4 °C, then centrifuged 15 min at 16,000 × g, dried on air, dissolved in 4× Laemmli sample buffer, and separated by 12% SDS-PAGE. Hsp25 in fractions was detected by Western blot and quantified with the AIDA program (Raytest Inc., Straubenhardt, Germany).

Glutaraldehyde Cross-linking—Cross-linking experiment was carried out as was described before (31): equal volumes and amounts of cell lysates were mixed with one volume of 0–4% glutaraldehyde in water. After incubation for 30 min at 30 °C,
the reaction was stopped by adding one volume of 1 M Tris-HCl containing 10% SDS and 10 mM EDTA. Aliquots were subjected to 5–15% SDS-PAGE. Cross-linked Hsp25 was detected by immunoblotting with antibody against Hsp25.

Immunocytochemistry—The cells were seeded on poly-L-lysine-coated coverslips and left overnight to attach. After treatment, cells were washed three times with PBS and fixed with 3.7% paraformaldehyde. F-actin was stained with TRITC-phalloidin.

Pulldown Experiments—After stimulation with arsenite cells were washed three times with ice-cold PBS and lysed. Lysates were incubated with 10 μg of GST-fused 14-3-3 or GST protein and 25 μl of glutathione beads overnight at 4 °C. Beads were washed four times with ice-cold IP buffer (Tris-buffered saline, 50 mM NaF, 1% Triton X-100, 1 mM Na3VO4) and subjected to 12% SDS gel followed by Western blot. Hsp25 was detected by anti-Hsp25 antibody.

Statistical Analysis—Data are presented as means ± S.E. Comparisons between wild type and MK2-deficient cells were performed using Student’s t test. A value of p < 0.05 was considered to be statistically significant.

RESULTS

p38- and MK2-dependent Phosphorylation of sHsps upon Stress Treatment by Arsenite—To analyze the role of phosphorylation of endogenous sHsp we first investigated the suitability of phospho-specific antibodies for Western blot detection of phospho-sHsps in lysates from mouse and human cells. For MEFs arsenite-stimulated phosphorylation of serine residue 86 (pSer86)-Hsp25 was detected by pSer86-specific Hsp27 (human homologue of mouse Hsp25) antibody that cross-reacts with mouse pSer86. B. HeLa cells were preincubated with inhibitors against p38 (SB203580) or MK2 (MK2-I) for 2 h and subsequently stimulated with arsenite (200 μM) for 1 h. Cells were lysed, and equal amounts of total protein were loaded to SDS gel. Phosphorylation of Hsp27 at Ser15 and Ser82 was visualized in Western blot by pSer15- and pSer82-specific antibodies, respectively. Total Hsp27 indicates equal loading. C. Activation of the p38 MAPK cascade and Hsp25 phosphorylation in WT and MK2-deficient MEFs. The Western blot experiment using phospho-p38 antibodies shows that arsenite stimulation (100 μM for 1 h) leads to p38 phosphorylation in both WT and MK2-deficient cells. However, p38-mediated Hsp25 phosphorylation at Ser86 can be detected only in WT MEFs, whereas the total amount of Hsp25 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reflects equal protein loading.

FIGURE 1. p38- and MK2-dependent phosphorylation of Hsp25. A, phosphorylation of Hsp25 S86 after arsenite (Ars) stimulation depends on specific inhibitor of p38 kinase SB203580. MEF cells were preincubated with SB203580 or its inactive analog, SB202474, for 30 min before stimulation with 100 μM arsenite for 1 h. Phospho-Ser86 (pS86)-Hsp25 was detected by pSer86-specific Hsp27 (human homologue of mouse Hsp25) antibody that cross-reacts with mouse pSer86. B, HeLa cells were preincubated with inhibitors against p38 (SB203580) or MK2 (MK2-I) for 2 h and subsequently stimulated with arsenite (200 μM) for 1 h. Cells were lysed, and equal amounts of total protein were loaded to SDS gel. Phosphorylation of Hsp27 at Ser15 and Ser82 was visualized in Western blot by pSer15- and pSer82-specific antibodies, respectively. Total Hsp27 indicates equal loading. C, activation of the p38 MAPK cascade and Hsp25 phosphorylation in WT and MK2-deficient MEFs. The Western blot experiment using phospho-p38 antibodies shows that arsenite stimulation (100 μM for 1 h) leads to p38 phosphorylation in both WT and MK2-deficient cells. However, p38-mediated Hsp25 phosphorylation at Ser86 can be detected only in WT MEFs, whereas the total amount of Hsp25 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reflects equal protein loading.

FIGURE 2. Analysis of phosphorylation-dependent Hsp25 oligomerization by gel filtration. A, after arsenite (Ars) stimulation (100 μM for 1 h) lysates were prepared from WT and MK2-deficient MEFs. A, cell lysates were separated on the Superose 6 column, and fractions were precipitated by trichloroacetic acid and analyzed for the presence of Hsp25 by Western blotting. B. results from three independent experiments were quantified using the AIDA quantification program (Raytest Inc.). The total amount of Hsp25 in the Western blot was taken as 100%, and the sum of signal intensity in high or low molecular mass fractions was expressed as a percentage from the total amount of Hsp25 in the blot. Data are presented as mean ± S.D. 99% of total Hsp25 in WT were found in low molecular mass fractions after arsenite stimulation compared with 45 and 44% in MK2 /− and controls, respectively (p < 0.05).
against the human homologue of the other MK2 phosphorylation site in Hsp25, serine 15, fail to detect the phospho-protein from mouse cells (data not shown). To understand whether there are general differences in stress-dependent phosphorylation of these two sites of sHsps, we analyzed Hsp27 phosphorylation at serine 15 and serine 82, the homologous site to Ser86 in mouse, in human HeLa cells (Fig. 1B). It can be seen that both sites are similarly phosphorylated in response to arsenite treatment and that this phosphorylation is almost completely inhibited by SB203580. Hence, we got the impression that analysis of Hsp25-pSer86 will be representative for stress-dependent phosphorylation of both sites.

In HeLa cells, we also used a newly developed inhibitor against MK2 and MK3, the compound 2,4-diamino-5H-chromeno-[2,3-b]pyridine-3-carbonitrile (U. S. Patent 2004/0127511), designated as MK2-I. In a concentration of 15 μM, this inhibitor, which has an IC50 of about 0.6 μM, also leads to a significant inhibition of Hsp27 phosphorylation at both sites analyzed. This clearly indicates the role of the p38 MAPK cascade including the downstream kinases MK2 and/or MK3 in sHsp phosphorylation.

After having shown that the anti-pSer86 antibody is well suited for monitoring Hsp25 phosphorylation in MEFs, we decided to use this antibody in genetically altered MEFs, which lack the protein kinase MK2 (29). While in WT cells an arsenite-dependent phosphorylation of Hsp25 can be detected, it is absent in MK2-deficient (MK2−/−) cells, although p38 is activated in both cell lines to a comparable degree (Fig. 1C). This supports the notion that MK2 is the major kinase that phosphorylates endogenous Hsp25 after arsenite treatment in mouse embryonic fibroblasts.

Analysis of Hsp25 Oligomerization in WT and MK2−/− MEFs upon Arsenite Treatment—Since arsenite-induced phosphorylation of Hsp25 is absent in MK2−/− MEFs, this cell line should be well suited to analyze the role of phosphorylation of endogenous Hsp25 for its oligomerization in vivo. First, size exclusion chromatography of lysates from non-stimulated and arsenite-treated WT and MK2−/− cells was performed (Fig. 2). We were able to detect two different peaks for Hsp25. Hsp25 exists both as oligomers up to about 430 kDa, corresponding to about 16–18 subunits, and as tetramer, dimer, or monomer in a mass range between 25 and 75 kDa (Fig. 2A).

In non-stimulated cells, Hsp25 is nearly equally distributed between high (660–75 kDa) and low (75–25 kDa) molecular mass fractions and there is no obvious difference detected between WT and MK2−/− (Fig. 2B). However, after arsenite treatment the amount of Hsp25 in high molecular mass complexes is significantly decreased in WT cells, whereas arsenite-treated WT and MK2−/− cells show no difference from control samples as also seen from quantification of three independent gel filtration experiments (Fig. 2B). Keeping in mind that Western blot is a semiquantitative method and that its quantification is lim-
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|          | GST-14-3-3 γ | GST |
|----------|--------------|-----|
| Ars      | -            | -   |
| SB203580 | -            | -   |
| MK2      | +/-          | +   |

**FIGURE 4.** Phosphorylation of Hsp25 enables its interaction with 14-3-3 proteins in vitro. Lyssates prepared from WT (MK2+/+) or MK2−/− MEFs preincubated with SB203580 for 30 min or left untreated before arsenite (Ars+) stimulation (200 μM for 1 h) and control lysates (Ars−) were incubated with recombinant GST-14-3-3-γ or as a control, with GST. After GST pull down, Hsp25 in the pull down (P) and in the supernatant (S) was detected by Western blot.

The interaction is p38-dependent, since SB203580 significantly decreased Hsp25 binding to 14-3-3-γ protein. The interaction is also MK2-dependent, since no Hsp25 was detected in pulldown experiments with 14-3-3-γ using lysates from arsenite-stimulated MK2−/− cells. Hsp25 had been detected in pull down with isoforms 14-3-3-β, ε, η, σ, τ, ζ in vitro in a MK2-dependent manner as well (data not shown), indicating that there is no strict isoform specificity for this interaction at least in vitro. However, in the in vitro binding assay less than 5% of phosphorylated Hsp25 is bound to the GST-14-3-3 proteins present in stoichiometric excess and the interaction needs to be confirmed in vivo.

**Effect of Heat Shock and Arsenite on Actin Cytoskeleton Disruption in WT and MK2−/− MEF Cells**—sHsps and their phosphorylation have been demonstrated to play a role in actin reorganization (20, 21, 37, 38). To examine the influence of MK2-mediated phosphorylation on the ability of endogenous Hsp25 to alter actin cytoskeleton in MEFs, actin filaments in control cells and cells treated either with heat shock or with arsenite were stained with TRITC-phalloidin. As shown in Fig. 5, after arsenite stimulation with a dose and for a time that were sufficient to cause remarkable phosphorylation of endogenous Hsp25 as well as changes in Hsp25 oligomer structure, no differences between WT and MK2−/− cells in regard to actin cytoskeleton can be detected after 2 h. Moreover, this concentration of arsenite did not cause any detectable changes in actin cytoskeleton in these cells after 2 h compared with non-treated control. However, after 3 h there is a lethal effect seen for WT cells which detach and round up, while MK2−/− cells are only slightly changed in morphology. As a control, lethal heat shock treatment at 45 °C leads to effects such as detachment and round up in both WT and MK2−/− cells. Apart from this, no visible differences could be detected between WT and MK2−/− cells in actin remodeling after PDGF treatment (data not shown). Taken together, it is unlikely that there is a direct effect of MK2-dependent Hsp25 phosphorylation on actin remodeling in the MEFs analyzed, but differences could exist in cellular resistance against arsenite.

**Delayed Insolubilization of Hsp25 in Stress-treated MK2-deficient MEFs**—It is also known that Hsp25/27 becomes detergent-insoluble (39) by binding to large cytoskeletal structures or by forming intracellular heat shock granules upon stress treatment (40). Therefore, we examined whether MK2 deficiency will affect the distribution of Hsp25 between soluble and insoluble fractions (Fig. 6). Treatment with arsenite (200 μM) for different times leads to increasing redistribution of Hsp25 into detergent-insoluble fraction in WT cells, whereas in MK2−/− cells the insolubilization is reduced and delayed (Fig. 6A). This is also seen when different arsenite concentrations (100 μM, 200 μM, Fig. 6B) and H$_2$O$_2$ treatment (20 μM and 60 μM for 1 h, Fig. 6C), as another oxidative stress, were used.

It is also known that MK2−/− the ratio between high and low molecular mass fraction stays without significant differences after stimulation with arsenite.

MK2-dependent Hsp25-14-3-3 Interaction in Vitro—14-3-3 proteins are expressed widely, are implicated in regulation of many processes in the cell, like cell viability and apoptosis, and interact with more than 200 proteins, predominantly after phosphorylation (33, 34). Since one of sHsps family members, Hsp20, had been recently identified as 14-3-3-binding protein (35), and since MK2 phosphorylation can generate 14-3-3-binding sites in case of other MK2 substrates (36), we asked whether Hsp25 interacts with 14-3-3 proteins in a MK2-dependent manner. Accordingly, recombinant GST-fused 14-3-3 proteins have been used for pulldown experiments. MEFs were untreated or stimulated with 200 μM arsenite for 1 h, lysed, and incubated with GST-14-3-3-γ. Hsp25 interacts with 14-3-3-γ after stimulation of the cell culture with arsenite but not in control cells (Fig. 4).
Phospho-Hsp25 can be detected in both soluble and insoluble fractions from WT cells, and with increasing insolubilization of total Hsp25 the level of insoluble phospho-Hsp25 increases in parallel. This indicates that phosphorylation per se is not responsible for insolubilization, but rather the changes of the oligomeric properties or intracellular interactions of Hsp25 are a result of phosphorylation.

Next, we analyzed sublethal (42 °C) and lethal (45 °C) heat shock treatment for different times (Fig. 6, D and E). Interestingly, a gradually increased insolubilization of Hsp25, which is reduced in MK2-deficient cells, is observed for sublethal heat shock conditions (Fig. 6D) while for lethal heat shock Hsp25 and phospho-Hsp25 are rapidly and almost completely translocated into the insoluble fraction, and no significant differences in Hsp25 redistribution from soluble into insoluble fraction between WT and MK2−/− cells were observed (Fig. 6E). This indicates that upon lethal heat shock, MK2-independent processes may overwrite the MK2-dependent insolubilization detected for oxidative stress and sublethal heat shock.

Delayed Hsp27 Stress Granule Formation in MK2−/− Cells after Arsenite Treatment—To see whether insolubilization is paralleled by stress granule formation, we examined subcellular localization of transfected EGFP-Hsp27 before and after arsenite stress in WT and MK2−/− cells (Fig. 7). While in control cells, EGFP-Hsp27 showed homogeneous cytoplasm localization; after treatment with arsenite at 200 μM for 2 h, we observed perinuclear or nuclear stress granule formation in WT cells. However, at this time of identical arsenite treatment, most MK2-deficient cells still display homogeneous cytoplasmic localization of EGFP-Hsp27. After 3 h of treatment, WT cells showed aggregation and morphological changes, indicating lethal effects, while MK2−/− cells are still normal in shape but now show increase in stress granule formation. The delay in stress granule formation observed corresponds to the delay in insolubilization in MK2-deficient cells and is probably due to the lack of phosphorylation of EGFP-Hsp27 by MK2.

MK2−/− Cells Show Higher Resistance against Oxidative Stress—By using overexpression of WT-sHsps and their phosphorylation mutants as well as by use of p38 inhibitors, it has been shown that phosphorylation of sHsps modulates cellular stress resistance (41–43). To examine how MK2-induced phosphorylation of endogenous Hsp25 will affect cellular stress resistance, we first measured cell survival after arsenite treat-
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MEFs were seeded into 96-well plates and treated with different concentrations of arsenite. After incubation for 48 h, where cells reach about 90% of confluence, cell viability was measured by monitoring cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in a colorimetric assay. An increased resistance of MK2-deficient MEFs at arsenite concentrations between 2 and 10 μM can be detected (Fig. 8A). This is in qualitative agreement with the observation that EGFP-Hsp27-transfected MK2-deficient MEFs still survive 3 h of 200 μM arsenite treatment, whereas WT cells show morphological signs of lethality (Fig. 7). We also determined the number of living cells after 1, 2, and 3 days of cultivation in medium containing 4 μM arsenite (inset to Fig. 8A). It can be seen that in the presence of arsenite MK2-deficient fibroblasts survive at least 1–2 days longer than WT cells without displaying significant proliferation.

To be sure that the differences between WT and MK2−/− are not a result of immortalization and cell line selection, we also analyzed arsenite resistance of primary MEFs prepared from WT and MK2-deficient embryos (Fig. 8B). Although the resistance of these cells against arsenite treatment is slightly higher than for immortalized MEFs, the result obtained is very similar to immortalized cells: MK2-deficient primary MEFs show increased resistance at arsenite concentration from 8 to 16 μM.

FIGURE 6. Stress-induced insolubilization of Hsp25 in WT and MK2−/− MEFs. A, analysis of Hsp25 redistribution from detergent-soluble to insoluble fraction after different times of arsenite (Ars) treatment. WT and MK2−/− MEFs were incubated with medium containing 200 μM arsenite for 0, 1, 2, and 3 h. After incubation cells were lysed and centrifuged for 15 min at 16,000 × g. Supernatant and pellet (re-suspended in RIPA buffer) were subjected to Western blot analysis using Hsp25- and pSer86-Hsp25-specific antibodies. B, dose-dependent phosphorylation and insolubilization of Hsp25. Arsenite was applied to MEFs for 1 h at 100 or 200 μM concentration, followed by fractionation and Western blot as described for A. C, phosphorylation and insolubilization of Hsp25 in response to H2O2 treatment (20 and 60 μM for 1 h). Fractionation and Western blot were the same as described for A, D and E, redistribution of Hsp25 from soluble into insoluble fraction in WT and MK2−/− MEFs as a result of mild heat shock (HS) of 42 °C for 1 h (D) and severe heat shock of 45 °C for 1 h (E). Fractionation and Western blot were the same as described for A.
To see whether the differences in resistance are stress-specific, we next used \( \text{H}_2\text{O}_2 \) as a different oxidative stress for primary MEFs (Fig. 8C). After 48 h of treatment with different concentrations of \( \text{H}_2\text{O}_2 \), a clearly increased stress resistance of MK2-deficient cells could be measured confirming the arsenite data. Since oxidative stress is long known to mediate apoptosis (44), we ask whether the increased resistance of MK2-deficient cells is due to decreased apoptosis. Caspase 3/7 activity was determined in arsenite- and \( \text{H}_2\text{O}_2 \)-treated cells (Fig. 8D). Indeed, the increased resistance of MK2-deficient cells against both arsenite and \( \text{H}_2\text{O}_2 \) is paralleled by significantly reduced levels of activated caspase 3/7.

**MK2 \(^{-/-} \) Cells Show Higher Resistance against Sublethal but Not against Lethal Heat Shock**—Since we observed differences in Hsp25 insolubilization in cells treated with sublethal and lethal heat shock (cf. Fig. 6, D and E), we were interested in resistance of fibroblasts against these heat shock conditions. MEFs were exposed to 42 or 45 °C for the times indicated. After PBS wash and addition of fresh culture medium, cell vitality was measured by the WST-1 conversion assay. Interestingly, under sublethal heat shock conditions, where a delayed insolubilization of Hsp25 is observed in MK2-deficient cells, after 4, 12, and 20 h of heat treatment these cells also display increased resistance compared with WT (Fig. 8E). In contrast, upon severe heat shock there is almost complete stress-killing of cells after 1 h and no significant difference between WT and MK2-deficient MEFs (Fig. 8F) supporting the notion that other MK2-independent processes determine cell damage under these lethal conditions.

**DISCUSSION**

The lack of Ser\(^{86} \) phosphorylation of Hsp25 in cells lacking MK2 activity strongly suggests that MK2 is the main kinase that phosphorylates Ser\(^{86} \) of endogenous Hsp25 in MEF cells *in vivo*. However, this finding does not exclude that other non-defined protein kinases in MEFs, which are directly or indirectly activated by MK2, are also involved in Hsp25 phosphorylation at Ser\(^{86} \). From this finding we also cannot exclude that other kinases, such as MK3 (14), MK5 (15), PKC\(^{\alpha} \) (16), and PKD (17) and PKG (13) can phosphorylate Hsp25 in a cell- or stress-type-specific manner at the same or different sites. At least in MEFs and in response to arsenite and heat shock, Ser\(^{86} \) of Hsp25 is phosphorylated exclusively by MK2. These results are in agreement with previous data from our laboratory, where two-dimensional phosphoprotein analysis revealed a lack of Hsp25 phosphorylation in MK2 \(^{-/-} \) cells but not in MK5 \(^{-/-} \) cells after lipopolysaccharide and arsenite stimulation (29, 45). Although MK3, another p38-activated kinase that appears to be very similar to MK2 in structure and substrate
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specificity (14, 46), was still present in MK2−/− cells, it seems that there is no biologically significant role of MK3 in Hsp25 phosphorylation in MEFs upon stress response.

The function of sHsp phosphorylation is still rather enigmatic. However, it is agreed that phosphorylation modulates sHsp function by changing its oligomerization, subcellular localization(s), and/or protein-protein interactions (47). The influence of phosphorylation on sHsp oligomerization has been studied using phosphorylation mutants of hamster and phospho-mimicking mutants of human Hsp27 (25, 31). From these studies it became clear that phosphorylation of overexpressed Hsp27 is necessary for disaggregation of the large oligomers. The involvement of the p38 MAPK pathway in disaggregation was demonstrated by the inhibitor SB203580 (26). Here we now demonstrate that phosphorylation by MK2 is necessary for disaggregation of endogenous Hsp25 oligomers *in vivo*. Hence, this confirms the overexpression studies and identifies MK2, one of several downstream targets of p38 MAPKα,β, as the enzyme responsible.

We demonstrate that in parallel to the lack of arsenite-, H2O2-, and sublethal heat shock-stimulated phosphorylation of Hsp25 a decreased and delayed accumulation of Hsp25 in the detergent-insoluble cell fraction takes place, indicating that under these stress conditions MK2-dependent disaggregation of sHsps is a prerequisite for its later insolubilization. However, similar insolubilization in WT and MK2−/− cells under lethal heat shock conditions implies further dominant MK2-independent mechanisms that determine insolubilization of Hsp25 in addition to sHsp phosphorylation and disaggregation.

We determined cell viability after arsenite, H2O2, and heat shock treatment and recovery and regard this as a measure of cellular stress resistance. The higher resistance of MK2−/− cells compared with WT cells in response to oxidative stress and sublethal heat shock is in agreement with the findings that overexpression of non-phosphorylatable mutants of Hsp25 can protect against oxidative stress (22), that overexpression of the phosphorylation mimicking mutant Hsp27-S15,78,82D cannot protect against oxidative stress (25), and that inhibition of Hsp25 phosphorylation by SB203580 does not inhibit protection against oxidative stress (22). These findings can be explained by the ability of large oligomeric complexes to control reactive oxygen species and glutathione levels (48, 49) and/or by the higher chaperone activity of the large oligomeric complexes (25). The higher resistance of MK2−/− cells to arsenite and H2O2 treatment correlates with the delay of insolubilization of Hsp25 and decreased activation of the pro-apoptotic caspases 3 and 7 indicating that control of reactive oxygen species may prevent apoptosis. In contrast, for the lethal heat shock conditions chosen, there is no significant stress resistance and almost complete Hsp25 insolubilization in both WT and MK2−/− cells. This indicates that the insolubilization observed in the above experiments is probably not the formation of productive stress granules (30) but rather represents non-productive and pro-apoptotic complex formation with irreversibly denatured proteins (4) resulting in irreversible damage of the stressed cells and/or a withdraw of Hsp25 from its anti-apoptotic functions (50), such as cytochrome c (51) or Daxx binding (52). Interestingly, the p38 MAPK cascade also exhibits a pro-apoptotic function (53), and p38-deficient fibroblasts are more resistant against stress stimuli, such as UV or serum deprivation (43).

We demonstrated *in vitro* binding of Hsp25 to 14-3-3 protein depending on MK2 activity, whereas other interactions of Hsp25, e.g. binding to Akt/PKB, are phosphorylation-independent.3 The phosphorylation-dependent binding is shared by other substrates of MK2, such as tristetraproline (54) and CDC25B and -C (55), in which MK2 generates binding motifs for dimeric 14-3-3 proteins. Furthermore, another sHsp, Hsp20, shows phosphorylation-dependent 14-3-3 binding, which competes with binding of coflin, an inhibitor of actin polymerization, to 14-3-3 (35). Although we were not able to detect Hsp25/14-3-3 protein complexes *in vivo* by co-immunoprecipitation so far, one may speculate that Hsp25/27 similarly competes with coflin in 14-3-3 binding in a phosphorylation-dependent manner resulting in actin remodeling (see also Ref. 36). Furthermore, it may well be that the pleiotrophic and homeostatic function of sHsps is based on competition with other 14-3-3 binding partners as well.

In the end, we should also mention the limitations of the MK2 knock-out approach chosen here for understanding the functional role of Hsp25 phosphorylation. First, it is known that due to stable complex formation between p38 and MK2 (56) the level of p38 is often significantly reduced in cell types lacking MK2 (57). To a certain, rather small degree this is also the case in the MEFs analyzed (cf. Fig. 1C). Hence, the possibility to see interfering effects of reduction of p38 level could not completely be excluded. Second, and more importantly, Hsp25 is not the only known substrate for MK2, but there is an increasing number of MK2 targets involved in regulation of cytoskeleton, cell proliferation, and migration, as well as gene expression at different levels (see Ref. 36). Some substrates of MK2, such as F-actin capping protein, Z-interacting protein (Cap-ZIP) (58), and the p16 subunit of the actin-related protein-2/3 complex (p16-Arc) (59), are also ubiquitously expressed and involved in actin remodeling. Hence, it can also not be excluded that the missing effect of the lack of Hsp25 phosphorylation on the actin filaments demonstrated here results from compensation of the effect by the lack of phosphorylation of Cap-ZIP and/or p16-Arc. Finally, the possible involvement of MK2 in cell cycle checkpoint control (55, 60) could also interfere with determination of cell proliferation and viability used to describe stress resistance.

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