Heat Shock Protein 27 Functions in Inflammatory Gene Expression and Transforming Growth Factor-β-activated Kinase-1 (TAK1)-mediated Signaling*

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Heat shock protein (HSP) 27 has long been known to be a component of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. p38 MAPK has important functions in the inflammatory response, but the role of HSP27 in inflammation has remained unknown. We have used small interfering RNAs to suppress HSP27 expression in HeLa cells and fibroblasts and found that it is required for pro-inflammatory cell signaling and the expression of pro-inflammatory genes. HSP27 is needed for the activation by interleukin (IL)-1 of TAK1 and downstream signaling by p38 MAPK, JNK, and their activators (MKK-3, -4, -6, -7) and IKKβ. IL-1-induced ERK activation appears to be independent of HSP27. HSP27 is required for both IL-1 and TNF-induced signaling pathways for which the most upstream common signaling protein is TAK1. HSP27 is also required for IL-1-induced expression of the pro-inflammatory mediators, cyclooxygenase-2, IL-6, and IL-8. HSP27 functions to drive cyclooxygenase-2 and IL-6 expression by augmenting the activation of the kinase downstream of p38 MAPK, MK2, resulting in stabilization of cyclooxygenase-2 and IL-6 mRNAs. The mechanism may not occur in cells of myeloid lineage because HSP27 protein was undetectable in human monocytes and murine macrophages.

Heat shock proteins (HSP) are constitutively expressed in certain cell types and are also induced upon exposure of cells to elevated temperatures and other cell stresses (1). HSP27 (or HSPB1) is a widely expressed 27-kDa protein and one of ten members of the small HSP family. These proteins include the lens proteins αA- and αB crystallin, and they all share a conserved C-terminal motif, the α-crystallin domain (2). The function of HSP27 is poorly understood, but recently missense mutations in the gene encoding it have been associated with the human neurodegenerative disorders, Charcot-Marie-Tooth disease, and distal hereditary motor neuropathy (3). However, the role of the HSP27 in these diseases is unclear.

HSP27 is phosphorylated in response to heat shock, cell stresses, pro-inflammatory stimuli, and various agonists including phorbol 12-myristoyl 13-acetate (PMA) (4–6). In fact HSP27 was found to be a major protein phosphorylated upon interleukin (IL)-1 treatment of fibroblasts (7). Phosphorylation of the human protein occurs at three serine residues Ser-15, Ser-78, and Ser-82 (6) and is performed by mitogen-activated protein kinase (MAPK)-activated protein kinase-2 (MK2) (8). *In vitro* phosphorylation of HSP27 by a kinase in cell lysates allowed the purification of an IL-1-activated protein kinase cascade now known as the p38 MAPK pathway (9). It consists of upstream activators (later identified as MAPK kinase (MKK)3 and MKK6 and Refs. 10 and 11), p38 MAPK, the downstream kinase, MK2, and HSP27 (9, 12). Phosphorylation of the protein by MK2 has been suggested to modulate different functional properties of HSP27, including oligomerization; the unphosphorylated protein exists as large ~700-kDa oligomers (e.g. 24-mers), which dissociate to form dimers upon phosphorylation (13–15).

Various cellular functions have been proposed for HSP27. It is cytoprotective against heat shock (16) and apoptotic agents including tumor necrosis factor (TNF) (17), Fas/APO-1, staurosporine (18), H2O2 (19), and anticancer drugs (20). HSP27 possesses chaperone-like activity and refolds denatured proteins, preventing their aggregation (21). Reduced glutathione-dependent chaperone activity against misfolded or oxidized proteins has been suggested to be responsible for the HSP27 protective role (22). Several other anti-apoptotic functions have also been proposed (23); however, at present, there is no common mechanism that explains the cytoprotective function of HSP27. HSP27 may also regulate cytoskeletal dynamics. Over-
expression of HSP27 stabilizes actin filaments, and this process appears to require phosphorylation (24, 25).

The p38 MAPK pathway, in addition to phosphorylating HSP27, plays a key role in the induction of many genes of the inflammatory response. An important function is to stabilize otherwise unstable mRNAs, thereby increasing their expression (26). The mechanism involves AU-rich elements (ARE) present in the 3′-untranslated regions (UTR) of mRNAs that direct instability but also allow for stabilization by p38 MAPK (27, 28). The process requires the downstream kinase, MK2 (27–29) and is thought to involve proteins that interact with the ARE and thereby regulate the decay of mRNA (26).

The fact that HSP27 is a substrate of MK2 makes it a candidate for involvement in the inflammatory response. Although its phosphorylation following IL-1 treatment of cells was first described some 20 years ago (30), its function in inflammation remains unknown. To examine the role of HSP27 in the inflammatory response we suppressed the expression of the protein in HeLa cells and human fibroblasts by RNA interference (RNAi).

We found that HSP27 is needed for the induction by IL-1 of COX-2 and IL-6 protein and mRNA. In HSP27-depleted cells, the inhibition of COX-2 and IL-6 mRNA was a result of instability of these mRNAs, which was caused by reduced MK2 activation. These findings led us to uncover an unexpected function of these mRNAs, which was caused by reduced MK2 activation.

HSP27 Regulates Pro-inflammatory Cell Signaling

Human fibroblasts were seeded in 6-well plates (5 × 10^4 cells per well) and cultured for 24 h. The cells were transfected with dsRNA (10 nm final concentration) using Lipofectamine 2000 (Invitrogen) according to the instructions supplied. Cells were re-transfected 24-h later in the same way. Cells were stimulated and harvested for the times indicated at 72 h after the initial transfection.

Western Blotting—Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride, 3 μg ml⁻¹ aprotinin, 10 μM E64, 2 μg ml⁻¹ pepstatin, 10 mM NaF, 5 mM sodium orthovanadate, 1 μM microcystin, 2 mM dithiothreitol), and protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were stained with rabbit anti-COX-2 antibody (Alexis), a mouse anti-α-tubulin antibody DM-1A (Sigma-Aldrich), a mouse monoclonal antibody against actin (Sigma), a rabbit antiserum raised against MKK3/6 (32), a rabbit antiserum to human HSP27 (7), a rabbit antiserum that recognizes both human HSP27 and murine HSP25 (Stressgen), a rabbit antiserum to the C-terminal peptide of p38 MAPK (33), a rabbit antiserum against TAK1 (sc-7162; Santa Cruz Biotechnology), and a rabbit antiserum against IkBα (sc-203; Santa Cruz Biotechnology). Rabbit anti-phospho-ERK1/2, rabbit anti-phospho-p38 MAPK, rabbit anti-phospho-MKK3/6 antibodies, and mouse anti-phospho-JNK1/2 monoclonal antibody were from Cell Signaling, and secondary antibodies coupled to horseradish peroxidase were from Dako. Protein detection was carried out by enhanced chemiluminescence (GE Healthcare).

Enzyme-linked Immunosorbent Assay (ELISA)—IL-6 and IL-8 concentrations in medium were determined by ELISA using a kit (BD Biosciences) according to the manufacturer’s instructions. A prostaglandin E2 (PGE2) ELISA kit was from R&D Systems and was used according to the instructions supplied. All samples were developed using the TMB peroxidase substrate system (Kirkegaard & Perry Laboratories), and the reactions were stopped using 1 M H2SO4. Absorbance was read on an ELISA Multiskan Biochromic plate reader (Labsystems).

Data were analyzed using Ascent software (Labsystems).

RNA Isolation and Northern Blotting—RNA was isolated from cells using an RNA Blood Mini kit (Qiagen) according to the manufacturer’s instructions. 8 μg of HeLa cell RNA was electrophoresed on denaturing formaldehyde/1% agarose gels with 0.41 M formaldehyde. RNA was capillary-transferred onto Hybond XL membranes (GE Healthcare) and fixed by UV cross-linking. For cDNA probes, membranes were prehybridized for 3 h and then hybridized overnight with [α-32P]dCTP-labeled cDNA probe at 42 °C in Ultrahyb (Ambion). Blots were then washed three times for 1 h at 52 °C with 2 × SSC/0.1% SDS, 1 × SSC/0.1% SDS, and 0.1 × SSC/0.1% SDS. For GAPDH riboprobe pre-hybridization, hybridization, and washes were carried out at 65 °C. RNA was visualized and quantified using an FLA-2000 phosphorimager (Fuji, Tokyo, Japan). The GAPDH riboprobe template was from BD Biosciences and prepared according to Ref. 34. The HSP27 probe was prepared using a fragment of pCMVFLAG/HSP27 plasmid (28) obtained by BamHI digestion. IL-6 and COX-2 probes have been described previously (35, 36).
**Cell Viability Assay**—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg ml⁻¹ final concentration) was added to 1.5 ml of cell culture medium, and cells were incubated for 4 h. 1.5 ml of 10% SDS in 10 mM HCl was then added, and cells were incubated at 37 °C overnight. Aliquots were transferred to a 96-well plate, and absorbance at 620 nm was measured using an ELISA Multiskan Biochromic plate reader (Labsystems).

**Kinase Assays**—For MK2 and p38 MAPK, these were performed as described previously (36). JNK was immunoprecipitated in an identical fashion but using an antiserum against long JNK2 (37). p38 MAPK and JNK assays were performed using either recombinant His-tagged MK2 or GST-ATF-2 substrates, respectively. MKK4 was immunoprecipitated using a rabbit antiserum (sc-964; Santa Cruz Biotechnology). MKK7 was immunoprecipitated with a previously described antiserum (37). MKK4 and MKK7 were immunoprecipitated with a buffer lacking deoxycholate but using protein G beads, and they were assayed using GST-JNK2 (K55R) as previously described (37). IKK was assayed for after immunoprecipitation with an anti-IKK antibody (764) in lysis buffer containing 400 mM NaCl. Immunoprecipitates were washed in the same buffer and assayed using GST-IκBα as substrate (gift from Michael Karin, University of California San Diego, La Jolla, CA). TAK1 was immunoprecipitated with a sheep antibody against TAB1 and assayed using recombinant MBP-MKK6 (both gifts from Mark Windheim, MRC Protein Phosphorylation Unit, University of Dundee) as described previously (38).

**RESULTS**

**HSP27 Is Required for the Induction of COX-2, IL-6, and IL-8 proteins by IL-1**—To investigate whether HSP27 is required for the regulation of inflammatory response genes, its expression was suppressed in HeLa cells by RNAi, and the induction by IL-1 of COX-2, IL-6, and IL-8 was measured. Cells were transiently transfected separately with six different siRNAs targeted to HSP27 (HSP27#1–6; 10 nM final concentration), a control scrambled dsRNA (Scr; 10 nM final concentration), or left untransfected (Untr) and incubated for 72 h. Cells were then treated with IL-1 (20 ng ml⁻¹) for 4 h. A, Western blot of COX-2, HSP27, and α-tubulin (as a loading control). The blot shown is representative of six different experiments. B, HeLa cells were transfected with Scr, HSP27#2, or HSP27#5, or left untransfected and treated with IL-1 as above. Bar chart shows IL-6 ELISA (mean and S.E. of four experiments) data plotted as a percentage of IL-6 protein secreted by untransfected cells treated with IL-1 (20 ± 8 ng ml⁻¹). C, IL-8 ELISA as in B. IL-1-treated untransfected cells secreted 46 ± 19 ng ml⁻¹ IL-8 protein. D, expression of HSP27 in different cell types. Lysates were prepared from different cell types (indicated), and 15 μg of total protein were electrophoresed on SDS-PAGE and Western-blotted for HSP27 with an antibody that recognizes both human HSP27 and its murine orthologue, HSP25. E, requirement of HSP27 for IL-1-induced COX-2 expression in human fibroblasts. Human fibroblasts were transfected with Scr or HSP27 siRNAs (10 nM final concentration) as indicated or treated with Lipofectamine 2000 (Mock), and incubated for 24 h. Cells were then transfected again with the same amount of each dsRNA, or treated with Lipofectamine 2000 only, and incubated for a further 48 h. Cells were treated with IL-1 for 4 h, harvested, and lysed. Lysates were blotted for COX-2, HSP27, and actin as a loading control. Similar results were obtained in two or more separate experiments for each panel (A–E).
untransfected cells and in cells transfected with the scrambled oligonucleotide (Fig. 1A). The induction of COX-2 protein by IL-1 was inhibited by four different siRNAs but not by HSP27#3 or HSP27#4 (Fig. 1A). It is possible that HSP27#3 exerts off-target effects that block the inhibition of COX-2 upon HSP27 depletion. Because HSP27#2 and HSP27#5 siRNAs were the most effective at depleting HSP27 protein, they were selected for subsequent experiments. The effect of depleting HSP27 on IL-1-induced PGE2 secretion was also examined. The amount of PGE2 secreted following a 4-h IL-1 treatment was inhibited by 47 and 60% by HSP27#2 and HSP27#5 siRNAs compared with untransfected cells (data not shown).

The amounts of IL-6 and IL-8 secreted into the culture medium in response to IL-1 were also measured (Fig. 1, B and C). These were reduced in cells in which HSP27 expression was suppressed: siRNAs HSP27#2 and #5 inhibited IL-6 secretion by 72 ± 8% and 81 ± 7%, respectively (Fig. 1B) and reduced IL-8 secretion by 65 ± 4% and 68 ± 9%, respectively (Fig. 1C).

**HSP27 Regulates Pro-inflammatory Cell Signaling**

![FIGURE 2. HSP27 regulates IL-1-induced COX-2 and IL-6 mRNA levels and stabilization.](image)

*Fig. 2.* HSP27 regulates IL-1-induced COX-2 and IL-6 mRNA levels and stabilization. A, HeLa cells were transfected with HSP27 siRNAs, Scr, or left untransfected (as in Fig. 1) and either left untreated or treated with IL-1 for 2 h. Cells were then harvested, lysed, and RNA was isolated. Northern blots for COX-2, IL-6, HSP27, and GAPDH mRNA (as a loading control) are shown. Data are representative of two independent experiments. B, cells were transfected (as above), treated with IL-1, and incubated for 1.5 h before addition of actinomycin D (final concentration 5 μg ml⁻¹) to block transcription. Cells were harvested at the times shown, and RNA was isolated and Northern-blotted as above. Graphs show COX-2 or IL-6 mRNA levels normalized to GAPDH mRNA levels for representative actinomycin D chase experiments. Data shown in each panel are representative of at least two independent experiments.
RNAi was used to test whether HSP27 functions in inflammatory gene expression in human fibroblasts. A single round of transfection by lipofection was found to be insufficient for strong depletion of HSP27 protein (data not shown). The cells were therefore transfected twice by lipofection, with an interval of 24 h between transfections, to improve depletion of HSP27. The suppression achieved by HSP27#2 after sequential transfection of the fibroblasts was still not as complete as in HeLa cells (compare Fig. 1, A and E) and was not sufficient to inhibit COX-2 expression (Fig. 1E). However in fibroblasts, HSP27#5 strongly suppressed HSP27 expression and, also inhibited the induction of COX-2 protein by IL-1 (Fig. 1E). Thus the pro-inflammatory function of HSP27 is not limited to HeLa cells but also occurs in human fibroblasts.

HSP27 Is Required for the Induction of COX-2 and IL-6 mRNA by IL-1—Suppression of HSP27 expression might inhibit the translation of COX-2 and IL-6 or reduce their mRNA levels. To test this, cells were treated with the siRNAs as before and were left unstimulated or stimulated with IL-1. The cells were harvested after 2 h, and RNA was isolated and examined by Northern blotting. IL-1 treatment induced COX-2 mRNA in untransfected and Scr-transfected cells to a similar extent (Fig. 2A). HSP27#2 and HSP27#5 inhibited COX-2 mRNA by 50 and 56%, respectively, compared with untransfected cells (Fig. 2A). Stronger inhibition was seen for IL-6 mRNA (68% inhibition for both siRNAs) (Fig. 2A). The inhibition of IL-1-induced IL-6 mRNA (Fig. 2A) reflected the reduction in IL-6 protein (Fig. 1B). Therefore, HSP27 does not appear to regulate IL-6 translation. These results indicate that HSP27 regulates either the transcription or the stability of these inflammatory transcripts. Northern blots were also performed to measure the degree of suppression of HSP27 mRNA (Fig. 2A). Both siRNAs suppressed HSP27 mRNA (HSP27#2: 65 ± 8%; HSP27#5: 71 ± 7%).

HSP27 has previously been reported to have a cytoprotective role. It was possible that the inhibition of expression of inflammatory proteins in response to IL-1 was caused by the death of cells in which HSP27 expression was suppressed. To check this, the viability of oligonucleotide-treated cells was measured by the MTT assay. In three experiments, Scr and HSP27#2 had no effect on cell viability, and only a small reduction was seen with HSP27#5 (14 ± 3%). Decrease in viability did not account for the differences in COX-2, IL-6, and IL-8 expression.

HSP27 Is Needed for Stabilization of IL-1-induced COX-2 and IL-6 mRNAs—Because HSP27 is a component of the p38 MAPK signaling cascade and this pathway has an important post-transcriptional role in inflammatory gene expression, we...
examined the stability of COX-2 and IL-6 mRNAs in HSP27-depleted cells. Cells were transfected with dsRNA as before, then stimulated with IL-1 for 1.5 h. Actinomycin D was added to block transcription, and cells were then incubated further and harvested at different times. RNA was isolated and examined by Northern blotting to measure mRNA decay rates. After 1.5 h of IL-1 treatment, COX-2 mRNA in Scr-transfected cells was stable; however, in HSP27-suppressed cells, it was reduced in amount and decayed rapidly (Fig. 2B). IL-6 mRNA behaved similarly (Fig. 2B). The stability of various other mRNAs (Bcl-x, GADD45, c-Fos, p21, Bax, Mcl-1, and L32) was unaffected by HSP27 depletion (supplemental Fig. S1) showing that of the mRNAs tested, the effect is not general and may be specific to COX-2 and IL-6 mRNAs.

Overexpression of HSP27 has previously been shown to protect against the cytotoxic effects of actinomycin D (39). To check whether actinomycin D affected the viability of cells in which HSP27 protein was suppressed, MTT assays were performed. Scr- and HSP27#2-transfected cells were left untreated, treated with IL-1 for 2 h, treated with actinomycin D for 1.5 h, or treated with IL-1 for 2 h followed by actinomycin D for 1.5 h. In two separate experiments, cell viability was unaffected by any of these treatments (data not shown). Thus the instability of COX-2 and IL-6 mRNAs in HSP27-suppressed cells was not caused by death of the cells, which might have been caused by actinomycin D.

**HSP27 Is Required for Activation of MK2 by IL-1**—It is possible that suppression of HSP27 protein (which is phosphorylated by MK2) was affecting upstream signaling by the p38 MAPK pathway. To examine this possibility, Scr or HSP27#5-transfected cells were stimulated with IL-1 for different times. Activation of MK2 was measured in lysates by immunoprecipitating it and assaying for its ability to phosphorylate recombinant HSP27 in vitro (Fig. 3). MK2 activity peaked at 30 min after IL-1 treatment and was inhibited by 56% by HSP27#5 siRNA (Fig. 3). Because MK2 activity is required for stabilization of COX-2 and IL-6 mRNAs, the reduced stability of these transcripts in HSP27-depleted cells is entirely consistent with inhibition of MK2 following HSP27 depletion.

**HSP27 Is Needed for Full Activation by IL-1 of p38 MAPK and JNK, but Not ERK**—Because HSP27 regulates MK2, the activation by IL-1 of its upstream activator, p38 MAPK, was measured in Scr and HSP27#5-transfected cells. p38 MAPK activity was measured by immunocomplex kinase assay using recombinant MK2 as substrate (Fig. 4A). p38 MAPK activity was maximal at 30 min after IL-1 stimulation and was inhibited by 59 ± 14% following HSP27 depletion (Fig. 4A). The reduction in p38 MAPK activity in HSP27 knockdown cells was not a result of reduced p38 MAPK expression as this was the same in Scr- and HSP27#5-transfected cells. JNK is a MAPK, which, like p38 MAPK, is strongly activated by inflammatory stimuli including IL-1. ERK, the first discovered MAPK, is typically activated by mitogens, but is also activated by IL-1 in many cells. To find out whether the effect of HSP27 depletion on signaling is limited to the p38 MAPK pathway or affects the other MAPK pathways, the activation of JNK and ERK was examined. JNK was immunoprecipitated from dsRNA-transfected cell lysates and assayed using recombinant ATF-2 protein as a substrate (Fig. 4B). As for p38 MAPK, JNK was strongly activated by IL-1 in Scr-transfected cells, peaking at 30 min IL-1 (Fig. 4B). Peak JNK activity was reduced by 64 ± 2% in HSP27-depleted cells compared with Scr-transfected cells (Fig. 4B). Thus the role of HSP27 in regulating signaling is not restricted to the p38 MAPK pathway.
ERK activation was assessed by Western blotting lysates with an antiserum that detects phosphorylated and activated ERK. Phosphorylation of ERK in Scr-transfected cells peaked at 0.5 h IL-1 as seen for the two other MAPKs (Fig. 4C). In HSP27-depleted cells, phosphorylation of ERK also peaked at 0.5 h and was induced by IL-1 to a similar degree compared with Scr-transfected cells (Fig. 4C). Phosphorylation of ERK (at 0.5 h post-IL-1) caused some of the ERK protein to shift in mobility on the gel (Fig. 4C, bottom panel). HSP27 has been previously suggested to form a complex with protein kinase-B (PKB) and to be required for its activation (40). PKB was phosphorylated in resting cells, and IL-1 treatment for 30 min resulted in only a small increase in its activation (data not shown). Activation of PKB was not affected by suppression of HSP27 expression (data not shown). Therefore, of the kinases tested thus far, HSP27 specifically regulates MK2, p38 MAPK, and JNK. To verify that the effects of HSP27 depletion on MAPK activity were caused by HSP27 depletion, an additional siRNA was employed. Activation of p38 MAPK, JNK, and ERK was analyzed by Western blotting with phosphospecific antibodies. For p38 MAPK and JNK, but not ERK, this was inhibited in cells transfected with both HSP27#2 and HSP27#5 (Fig. 4D). The inhibition of p38 MAPK and JNK is not an off-target effect associated with a single siRNA.

HSP27 Is Required for Activation of MKK3/MKK6 and MKK4/MKK7—It was possible that HSP27 directly targets p38 MAPK and JNK or, alternatively, it could regulate upstream signaling. To test the latter possibility, the activation of the upstream activators of p38 MAPK and JNK was analyzed. Activation of MKK3 and MKK6, the kinases directly upstream of p38 MAPK, was detected by Western blotting with a phosphospecific antibody that recognizes activated forms of both kinases (Fig. 5A). As for MK2 and p38 MAPK, activation of MKK3 and MKK6 peaked at 30 min IL-1 and was inhibited upon suppression of HSP27 expression (Fig. 5A). The reduced signal for phospho-MKK3 and -MKK6 in HSP27 knockdown cells was not caused by a decrease in expression of these kinases, as this was normal in the HSP27-depleted cells (Fig. 5A). These results prompted us to examine the activation of the kinases responsible for JNK activation, MKK4 and MKK7.

These two kinases were immunoprecipitated and assayed for their ability to phosphorylate a kinase-dead mutant form of recombinant JNK protein. This mutant kinase (JNK K55R) does not undergo autophosphorylation during the assay, allowing MKK activity to be measured. IL-1-induced MKK4 activity, which peaked at 30 min post-IL-1 (Fig. 5B). Unlike the activation of the other IL-1-regulated kinases examined, which declined rapidly after their peak at 30 min, MKK7 activity was more sustained, with similar activity detected at 30 min and 1 h IL-1 (Fig. 5C). Peak MKK4 and MKK7 activity was lower in HSP27 knockdown cells by 65 and 60%, respectively (Fig. 5, B and C). The inhibition of MKK4 and MKK7 upon depleting HSP27 fully accounts for the inhibition of JNK. The amount of MKK4 and MKK7 protein in cells was found to be unchanged following HSP27 depletion (data not shown).

HSP27 Regulates IKKα Activation but Not IκBα Degradation—The p38 MAPK and JNK pathways are both strongly activated by pro-inflammatory stimuli, and the degree of activation depended upon HSP27. Another pathway that plays a pivotal role in the expression of inflammatory mediator genes is that of activating the nuclear factor (NF)-κB. It is possible that this is also regulated by HSP27. The transcription factor NF-κB is held in the cytoplasm of resting cells by an interaction with an inhibitor of NF-κB, α, the major form of which is IκBα. Upon cell activation, IκBβ phosphorylates IκBα, triggering its degradation and releasing NF-κB, which translocates to the nucleus to activate transcription. IκBβ activity and IκBα degradation were analyzed in HSP27-depleted cells.

IκBβ exists in a complex with IKKα and IKKγ (41). Activation of IκBβ was measured by immunoprecipitating IκBγ and assaying phosphorylation of recombinant IκBα (Fig. 6A). IκBβ was activated by IL-1, with activity peaking at 15 min post-IL-1 (Fig. 6A). HSP27 depletion inhibited IκBβ activity by 51% at 15 min IL-1 (Fig. 6A). Thus HSP27 regulates the activity of three IL-1-activated protein kinase cascades, namely the p38 MAPK, JNK, and IκBβ pathways, to similar extents. Despite inhibition...
of IκBα upon HSP27 knockdown, IL-1 induced normal degradation of IκBα in HSP27 siRNA-transfected cells (Fig. 6B).

**HSP27 Regulates TAK1 Function**—IL-1 activates the p38 MAPK, JNK, and NF-κB pathways via a common activator, transforming growth factor-β-activated kinase-1 (TAK1) (42). In cells, TAK1 exists in a complex with its binding partners TAK1-binding protein (TAB)1, TAB2, and TAB3. The complex was immunoprecipitated from lysates with an antibody against TAB1 and assayed for its ability to phosphorylate recombinant TAK1-binding protein (TAB)1, TAB2, and TAB3. The complex was not detected, and this was not regulated by HSP27 (Fig. 7). In resting cells, significant TAK1 activity could be detected, and this was not regulated by HSP27 (Fig. 7). TAK1 activity peaked at 10 min post-IL-1 in Scr- and HSP27 siRNA-transfected cells (Fig. 7). IL-1-induced TAK1 activity was inhibited by 53 ± 4% and 30 ± 7% at 5 and 10 min IL-1, respectively (Fig. 7). HSP27 depletion did not affect the amount of TAK1 protein in the cells (Fig. 7). Thus HSP27 activates pro-inflammatory signaling pathways by augmenting the function of TAK1.

It is generally accepted that TAK1 regulates the JNK, p38 MAPK, and NF-κB pathways. It was therefore surprising that IκBα degradation was unaffected by HSP27 depletion (Fig. 6B). To investigate whether TAK1 regulates IκBα degradation in HeLa cells, TAK1 expression was suppressed by RNAi (supplemental Fig. S2). TAK1 siRNA suppressed TAK1 protein levels and strongly inhibited IL-1-induced IκBα activity, but had little effect on IκBα degradation (supplemental Fig. S2).

**HSP27 regulates a signaling event common to TNF and IL-1**—TNF and IL-1 both activate the MAPK and NF-κB pathways via activation of TAK1 (42). However, these two cytokines activate TAK1 by different upstream mechanisms. IL-1 signaling involves MyD88, IRAK1, IRAK4, and TRAF6 (41), whereas IL-8. In the case of COX-2 and IL-6, we found that HSP27 regulated expression of the mRNAs. This was, at least in part, because of regulation of the stability of these mRNAs in IL-1-treated cells. HSP27 depletion had no effect on cell viability. Its effect on mRNA stability was not general and may be specific to mRNAs of inflammatory mediators such as COX-2 and IL-6. It is possible that IL-8 transcripts are regulated in a similar way.

COX-2 and IL-6 mRNAs are known to be stabilized upon p38 MAPK activation (36, 43). Reporter mRNAs bearing either the COX-2 (28), IL-6, or IL-8 (27) AREs are stabilized by co-expression of an active mutant of MK2 in a HeLa cell line. IL-6 mRNA is also unstable in lipopolysaccharide-treated macrophages from MK-2-null mice (29). We found that HSP27 is needed for activation of both p38 MAPK and MK2. Thus the regulation of p38 MAPK/MK2-mediated mRNA stabilization represents a mechanism whereby HSP27 contributes to the expression of pro-inflammatory mediators.

Regulation of pro-inflammatory cell signaling by HSP27 is not limited to the p38 MAPK pathway. HSP27 also functions in the activation by IL-1 of the JNK and NF-κB pathways. Reduced activity in these pathways in HSP27 knockdown cells was due to a defect in the function of the upstream activator, TAK1. TAK1 was originally thought to be involved in transforming growth factor-β signaling. It is now known to play an important role in signaling induced by a range of pro-inflammatory stimuli in fibroblasts (42), B cells (42), and T cells (44–46). TAK1 knock-out is lethal (42). Embryonic fibroblasts from TAK1-null mice display impaired activation by IL-1 and TNF of the p38 MAPK, JNK, and NF-κB pathways (42). Depletion of TAK1 by RNAi in HeLa cells also inhibits IL-1- and TNF-induced TNF signaling requires TRADD, TRAF2, and TRAF5 (41). To investigate whether HSP27 regulates signaling that is restricted to IL-1, or is common to both IL-1 and TNF, the effect of HSP27 depletion on TNF-induced JNK activation was examined (Fig. 7B). As seen for IL-1, TNF-induced JNK activity was also inhibited following HSP27 depletion (Fig. 7B). Thus TAK1 is the most upstream component common to both pathways that is targeted by HSP27.

**DISCUSSION**

We found that HSP27 functions in IL-1-induced cell signaling and pro-inflammatory gene expression. HSP27 is needed for full activation by IL-1 of TAK1 and signaling by the p38 MAPK, JNK, and IKKβ pathways. It is also required for the induction by IL-1 of three inflammatory mediators whose expression is known to be dependent on these pathways, namely COX-2, IL-6, and IL-8. In the case of COX-2 and IL-6, we found that HSP27 regulated expression of the mRNAs. This was, at least in part, because of regulation of the stability of these mRNAs in IL-1-treated cells. HSP27 depletion had no effect on cell viability. Its effect on mRNA stability was not general and may be specific to mRNAs of inflammatory mediators such as COX-2 and IL-6. It is possible that IL-8 transcripts are regulated in a similar way.

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NF-κB activation (47) and TNF-induced p38 MAPK and JNK activation (48).

HSP27 may act directly on TAK1, or could be required for its activation by upstream mechanisms. Our data suggest the latter because TAK1 activity was not regulated in unstimulated cells and was only regulated at early times following IL-1 treatment. MyD88, IRAK-1, and IRAK-4 are involved in signaling proximal to the IL-1 receptor. It is unlikely that HSP27 regulates these proteins, because they do not participate in signaling in response to TNF, which is also regulated by HSP27. Another difference between IL-1 and TNF signaling is that the IL-1 receptor interacts with TRAF6, whereas that for TNF binds TRAF2 and TRAF5. TRAF6 is a ubiquitin ligase, and TRAF2 has been shown to have ubiquitin ligase activity in vitro (49). They may both catalyze their autopolyubiquitination by Lys-63-linked ubiquitin chains (49). TAB2 and TAB3 then bind to these chains, initiating formation and activation of the TAK1 complex (50). HSP27 might play a role in ubiquitination mediated by TRAF6 and TRAF2. It will be interesting to test this in the future.

HSP27 regulates p38 MAPK, JNK, and IKKβ. It is thus possible that in addition to regulating mRNA stability, the small heat shock protein also activates transcription. In cells, IKK phosphorylates and activates the AP-1 transcription factors c-Jun and ATF-2 (51, 52). It is possible that transcription driven by AP-1 binding is regulated by HSP27. Despite regulation of IKKβ activity upon HSP27 depletion, the degradation of IkBα induced by IL-1 was not regulated. TAK1 depletion also inhibited IKKβ activity with almost no effect on IkBα degradation. Cells from TAK1−/− mice do show an impairment in NF-κB-driven transcription (42). The lack of an effect of either HSP27 or TAK1 siRNA on IkBα degradation is likely to be a result of residual IKKβ activity. Thus it is possible that HSP27-null cells would also show a defect in NF-κB-regulated transcription.

Phosphorylation causes dissociation of HSP27 multimers and may regulate the proposed function of HSP27 as a chaperone. It is unclear whether phosphorylation of HSP27 is required for it to promote signaling. The kinetics of HSP27 phosphorylation following IL-1 stimulation are similar to those for the activation of MK2 and p38 MAPK. TAK1 activity is regulated by HSP27 at 5 min following treatment of cells with IL-1. It is unclear to what extent HSP27 is phosphorylated at this time, because MK2 and p38 MAPK activity peak later (at 30-min post-IL-1).

HSP27 is known to have a cytoprotective role, and may also be involved in modeling of the actin cytoskeleton. Both of these processes could be regulated by HSP27 as a consequence of its function in cell signaling and gene expression. Cellular stresses, including heat shock, activate the p38 MAPK pathway, and HSP27 could contribute to signaling induced by these stimuli. Modeling of the actin cytoskeleton necessary for cell migration is regulated by LIM kinase, a kinase activated by MK2 (53). Thus the function of HSP27 may not be limited to pro-inflammatory signaling and the expression of only pro-inflammatory genes.

We have shown that HSP27 is required for pro-inflammatory gene expression in HeLa cells and human fibroblasts. The function of HSP27 may be to sensitize these cells to pro-inflammatory stimuli by augmenting pro-inflammatory signaling. It is likely that HSP27 plays a similar role in other cell types. It does not appear to be constitutively expressed in leukocytes, nor is it induced by lipopolysaccharide treatment of human monocytes or macrophages. It is perhaps rather surprising that these cells do not require HSP27 for pro-inflammatory signaling. The absence of HSP27 in hemopoietic cells suggests that it may function in innate, but not adaptive immunity.

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