The Function of HSP72 in Suppression of c-Jun N-terminal Kinase Activation Can Be Dissociated from Its Role in Prevention of Protein Damage*

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Activation of the c-Jun N-terminal kinase (JNK) by a variety of stimuli is critical for regulation of many cellular processes including apoptosis. The major inducible heat shock protein Hsp72 has previously been demonstrated to inhibit activation of JNK in cells exposed to heat shock and other protein-damaging agents, thus suppressing apoptosis. Hsp72 can protect proteins from stress-induced damage. To test if this protective function of Hsp72 is involved in JNK suppression, we investigated whether Hsp72 can avert activation of JNK by stimuli that do not cause protein damage. We show that Hsp72 suppresses activation of JNK induced by non-protein-damaging stimuli, interleukin-1 and UV irradiation, as well as by constitutively active components of the JNK signaling cascade Cdc42 and MEKK1. Furthermore, Hsp72 strongly reduced activation of JNK by phosphatase inhibitors. We also demonstrate that an Hsp72 mutant that lacks the ATPase domain is still capable of JNK suppression, thus indicating that the protein refolding activity of Hsp72 is not critical for inhibition of JNK activation. Taken together these data suggest that Hsp72 plays a regulatory role in JNK signaling and that the function of Hsp72 in protein protection or refolding is not involved in JNK regulation.

Mammalian cells respond to various environmental stimuli, such as UV irradiation heat shock, and cytokines (e.g. tumor necrosis factor and IL-1β) by activating the stress kinases JNK (c-Jun N-terminal kinase) and p38 (1). These protein kinases are involved in the regulation of cell proliferation, inflammation, immune responses, and other processes (2–4). In many cell types JNK activation has been shown to be essential for the transduction of a signal that leads to programmed cell death (apoptosis) in response to heat shock, UV irradiation, anticancer drugs, and certain other stimuli (5–9). Indeed, inhibition of JNK activation, as well as by constitutively active components of the JNK signaling cascade Cdc42 and MEKK1. Furthermore, Hsp72 strongly reduced activation of JNK by phosphatase inhibitors. We also demonstrate that an Hsp72 mutant that lacks the ATPase domain is still capable of JNK suppression, thus indicating that the protein refolding activity of Hsp72 is not critical for inhibition of JNK activation. Taken together these data suggest that Hsp72 plays a regulatory role in JNK signaling and that the function of Hsp72 in protein protection or refolding is not involved in JNK regulation.

There are two major isoforms of JNK in nonneuronal cells, JNK1 and JNK2 (referred as JNK), that are regulated in a similar manner. The pathway of JNK activation by UV and cytokines involves activation of small GTP-binding proteins including Cdc42, Rac, and Ras (11, 12). The signal is then transmitted to serine/threonine kinase MEKK1 (13) that phosphorylates and activates dual specificity kinase SEK1 (14) which, in turn, phosphorylates threonine and tyrosine residues in JNK (15), resulting in its activation (16). In contrast, activation of JNK by heat shock and certain other stressful agents proceeds via a distinct pathway that involves inhibition of a putative JNK phosphatase (17).

Previously we reported that in cells with elevated levels of the major heat shock protein, Hsp72, activation of JNK by heat shock and ethanol is strongly inhibited (10, 18). There are two alternative hypotheses that explain the Hsp72-mediated suppression of JNK activation by protein-damaging stresses. First, Hsp72, acting as a chaperone, prevents damage or promotes refolding of a critical JNK regulator that is inactivated upon protein-damaging treatments. The alternative hypothesis is that Hsp72 has a special regulatory function in the JNK signaling pathway. To distinguish between these possibilities, we addressed the question of whether Hsp72 can suppress activation of JNK by stimuli that do not cause protein damage. We also investigated whether suppression of JNK activation requires the ATPase activity of Hsp72, which is essential for its protein refolding function. In these experiments we employed a C-terminal fragment of Hsp72, which retains the capability to associate with unfolded proteins but cannot dissociate from such complexes due to the lack of the ATPase domain.

MATERIALS AND METHODS

Antibodies—In this study we used antibodies against the following: JNK1 (C-17) and GST (Z-5) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); phospho-SEK1 (Thr-223) (New England Biolabs, Beverly, MA); phospho-JNK (Promega); Hsp72 (SPA-810), Hsp72/Hsc75 (SPA-820), and Hsp40 (SPA-400) (StressGen Biotechnologies Corp., Victoria, Canada); HA epitope (monoclonal HA.11) (Berkeley Antibody Co., Richmond, CA).

Cell Lines—The monkey kidney COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Human lung fibroblasts IMR90 (passages 10–15) were grown in minimum Eagle’s medium supplemented with 20% fetal bovine serum and 2 mM glutamine. Cells were grown at 37 °C in an atmosphere of 5% CO2 to 60–85% confluence.

Plasmids—For transient transfection of COS-7 cells we used plasmids expressing JNK1 tagged with HA epitope (JNK1-HA) (19), a dominant negative mutant of SEK1 fused in frame to GST, GST-SEK1 (K/R) (15), a constitutively active mutant of Cdc42, Cdc42V12 (12), and a constitutively active N-terminal deleted mutant of MEKK1, MEKK1Δ (20). These plasmids were kindly provided by Dr. J. Avruch.

Transient Transfection of COS-7 Cells—COS-7 cells were transfected by a standard calcium phosphate technique. Briefly, cells were grown...
on 35-mm plates to 30–50% confluence, and 10 μg of total plasmid DNA with calcium phosphate solution was added to each plate. Corresponding vectors of plasmids were used for mock transfection and as a carrier when the amount of transfected plasmid of interest was lower than 10 μg. After overnight incubation cells were washed and incubated in growth medium for an additional 24–30 h to reach maximal expression of plasmid DNA. Efficiency of transfection was evaluated by fluorescence-activated cell sorter analysis using Coulter Epics Flow Cytometry Work Station, version 1.05.

**Adenovirus-based Expression of Hsp72**—A recombinant adeno virus vector-expressing Hsp72 (AdTR65Hsp72-GFP) was constructed by cloning a dicistronic transcription unit encoding human Hsp72 and the *Aequorea victoria* green fluorescent protein gene, separated by encephalomyocarditis virus internal ribosome entry site from pTR-DC/ HSP70-GFP (21, 22), into an adenovirus transfer vector. Expression of this transcription unit is controlled by the tetracycline-regulated transactivator protein tTA (23), which we expressed from the separate recombinant adeno virus (ADCMVTTA (24)). Twenty four hours after infection, the media were changed, and the cells were left for an additional 12 h. Inoculation of the cells with 3 × 10⁶ plaque-forming units of each virus per 35-mm dish was sufficient to infect almost 100% of the cells. This was confirmed each time by observation under a fluorescent microscope of a fraction of the cells expressing the green fluorescent protein. Thirty six hours after infection the cells accumulated large amounts of Hsp72 as was confirmed by immunoblotting of cytosol extracts with anti-Hsp72 antibody (not shown).

**Preparation of Cytosol Extracts and Analysis of JNK Activity**—Cells were washed twice with phosphate-buffered saline on a dish, aspirated, and lysed in 200 μl of lysis buffer per 35-mm dish (40 mM HEPES, pH 7.5; 50 mM KCl; 1% Triton X-100; 2 mM dithiothreitol, 1 mM Na₃VO₄; 50 mM β-glycerophosphate; 50 mM NaF; 5 mM EDTA; 5 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM benzamidine; 5 μg/mL each leupeptin, pepstatin A, aprotinin). The lysates were clarified by centrifugation in a microcentrifuge at 15,000 rpm for 5 min. Total protein concentration was measured in the supernatants with Bio-Rad protein assay reagent after which lysates were diluted with lysis buffer to achieve equal protein concentration in all samples. All procedures were performed at 4 °C.

For analysis of the state of phosphorylation of endogenous SEK1, extracts were subjected to immunoblotting with anti-phospho-SEK1 antibody. To assess the state of phosphorylation of transfected GST-SEK1, it was isolated from extracts with glutathione-agarose (Sigma), and precipitates were subjected to immunoblotting with anti-phospho-SEK1 antibody. Secondary antibodies conjugated with peroxidase were visualized with ECL substrates (Amersham Pharmacia Biotech), and films were quantified by densitometry.

To measure endogenous JNK activity, 5-μl aliquots of extract were added to a reaction mixture (20 μl final volume) containing (final concentration) the following: 40 mM HEPES, pH 7.5; 1 mM Na₄VO₄; 25 mM β-glycerophosphate; 10 mM MgCl₂; 20 μM ATP, 15 μCi of [γ-³²P]ATP and 40 ng of recombinant GST-c-Jun. The reaction was allowed to proceed for 25 min at 30 °C and then was stopped by addition of 10 μl of loading SDS-PAGE buffer. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to a Molecular Imager for quantitation. Subsequently, membranes were immunoblotted with an anti-JNK1 antibody to verify equivalent protein loading. All experiments were repeated at least three times.

To measure the activity of transfected JNK1-HA it was immunoprecipitated from extracts with antibody against HA epitope for 2 h at 4 °C. Precipitates were washed 3 times with a kinase reaction buffer: 25 mM HEPES, pH 7.4, 10 mM MgCl₂, 2 mM dithiothreitol, 25 mM β-glycerophosphate, 2 mM Na₄VO₄. Phosphorylation of GST-c-Jun by immunoprecipitated JNK was carried out in the same buffer in the presence of 20 μM ATP and 25 μCi of [γ-³²P]ATP at 37 °C for 10 min. The samples were then subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane and autoradiography. This membrane was later used for an immunoblot with anti-JNK1 antibody to ensure that equal amounts of the kinase were immunoprecipitated.

**RESULTS**

**Hsp72 Prevents Activation of JNK in Response to IL-1 and UV Irradiation**—To address the question whether Hsp72 can suppress activation of JNK induced by stimuli that do not cause protein damage, JNK was activated either by a proinflammatory cytokine IL-1 or by UV-C irradiation. Human fibroblasts were infected with adenovirus encoding Hsp72 under the control of a tetracycline-inhibitable promoter. In this expression system Hsp72 and green fluorescence protein (GFP) were encoded in a dicistronic transcription unit so that accumulation of GFP serves as a marker of Hsp72 synthesis (see “Materials and Methods” for details). Fluorescence microscopy of cells cultured without tetracycline for 36 h post-infection showed that almost 100% of the cells accumulated GFP, indicating that practically all cells expressed Hsp72. In control cell cultures grown in the presence of tetracycline no GFP fluorescence was detected, which indicates that Hsp72 was not expressed under these conditions. Indeed, expression of Hsp72 in cells grown in the absence of tetracycline and the lack of Hsp72 in control cells was confirmed by immunoblotting with anti-Hsp72 antibody (data not shown). Control and Hsp72-expressing cells were incubated with IL-1 for 5, 15, or 30 min. In control fibroblasts this treatment led to a transient, approximately 10-fold activation of JNK. However, in Hsp72-expressing cells JNK activation by IL-1 was suppressed (Fig. 1, A and B). Note that the expression of JNK was not influenced by adenoviral expression of Hsp72 (Fig. 1A). Even stronger Hsp72 inhibited JNK activation induced by UV irradiation. Treatment of control fibroblasts with UV (400 J/m²) resulted in rapid activation of JNK. Within 10 min, this kinase became activated 4–6-fold, and this level was maintained for at least the next 20 min. In contrast, in fibroblasts with elevated levels of Hsp72 UV activation of JNK was only about 2-fold (Fig. 2, A and B).

To confirm that exposure of fibroblasts to either UV or to IL-1 does not cause significant protein damage, we tested whether these stimuli induce a heat shock response, a general indicator of polypeptide damage in the cell. Fibroblast cultures were exposed to UV-C (400 J/m²) or IL-1 (20 ng/ml), and after 18 h induction of endogenous heat shock proteins, Hsp72 and Hsp40, was examined. In contrast to protein-damaging stress,
Function of HSP72 in Suppression of JNK Activation

Fig. 2. Hsp72 specifically inhibits JNK activity stimulated by UV irradiation of human fibroblasts. A, effect of high levels of Hsp72 on activation of JNK by UV irradiation. Fibroblasts were incubated with adenovirus as described in Fig. 1A and then were irradiated with 400 J/m² UV-C. After the indicated times, JNK activity was measured in lysates as described under “Materials and Methods.” B, quantitation of JNK activity measured in three independent experiments (mean ± S.E.). Experiments were performed as described in A and quantitated using a PhosphorImager. C, effect of Hsp72 on phosphorylation of SEK1 triggered by UV irradiation. Fibroblasts were incubated with adenovirus encoding Hsp72 as described in Fig. 1A and then were irradiated with 400 J/m² UV-C. After the indicated times, the levels of phosphorylation of endogenous SEK1 were assayed by immunoblotting of cellular lysates with anti-phospho-SEK1 antibody. D, effect of IL-1 and UV on induction of heat shock proteins. Fibroblasts were subjected to either heat shock at 45 °C for 45 min or UV-C irradiation at 45 °C for 45 min followed by recovery for 18 h, which caused strong induction of Hsp72 (Fig. 2D). In these cells JNK activation by UV irradiation was strongly suppressed as compared with nonpretreated control cells (Fig. 3, A and B). Heat shock pretreatment did not influence the level of JNK expression (data not shown). Similarly, endogenous Hsp72 induced by mild heat shock pretreatment suppressed activation of JNK by IL-1 (Fig. 3, C and D). These data further indicate that Hsp72 at elevated levels is able to inhibit activation of JNK by stimuli that do not cause protein damage.

Hsp72 Suppresses Activation of JNK Induced by Upstream Components of JNK-signaling Pathway—Although neither UV irradiation nor IL-1 activate heat shock response, there is still a remote possibility that these stimuli could cause specific damage of a critical JNK regulator (e.g. by production of reactive oxygen species), and Hsp72 may protect this component. Therefore, we tested if Hsp72 inhibits activation of JNK by constitutively active mutant forms of upstream elements of JNK-signaling pathway, Cdc42 and MEKK1 (see Fig. 7).

The following experiments were performed in COS-7 cells because transfection efficiency of primary fibroblasts is very low. COS-7 cells were transiently transfected with the plasmid-expressing Hsp72 under the control of a strong constitutive cytomegalovirus promoter. Cells transfected with empty vector were used as a control. Immunoblotting of cellular lysates showed about 2-fold increase in the levels of Hsp72 in cell cultures transfected with the Hsp72 plasmid compared with cells transfected with the empty vector. Considering 15% transfection efficiency (calculated from fluorescence-activated cell sorter analysis of cells transfected with GFP-expressing plasmid (data not shown)), successfully transfected cells contained about 7 times more Hsp72 than naive cells. To follow JNK activity in transfected cells only, cells were cotransfected with the plasmid-expressing JNK1 tagged with hemagglutinin (JNK1-HA), and JNK1-HA activity was assayed after immunoprecipitation with monoclonal anti-HA antibody from cellular lysates.

Initially, we tested whether Hsp72 is able to suppress JNK activation by IL-1 in transfected COS-7 cells, as was observed with fibroblasts (Fig. 1, A and B). Control and Hsp72-expressing cells were transfected with IL-1 for 20, 30, or 40 min. This treatment resulted in strong transient activation of JNK1-HA, which was markedly reduced in Hsp72-expressing cells (Fig. 4A). Cotransfection with Hsp72 did not affect the expression of JNK1-HA, as can be seen on the immunoblot of immunoprecipitated JNK1-HA probed with anti-JNK1 antibody (Fig. 4A). These data confirm that at elevated levels Hsp72 can inhibit activation of JNK by IL-1 and that COS-7 cells can be employed as a suitable model for further experiments.

To investigate the effect of Hsp72 on JNK activation by Cdc42, COS-7 cells were transiently transfected with the constitutively active mutant form of Cdc42 (Cdc42V12) along with JNK1-HA, and JNK1-HA activity was measured 48 h later. Transfection of cells with Cdc42V12 led to 4–5-fold activation of JNK1-HA in control cells. However, in cells expressing phosphorylated form of SEK1. As described above, activation of JNK in UV-irradiated cells expressing Hsp72 was markedly suppressed (Fig. 2, A and B). In contrast, under the same conditions SEK1 remained active (compare Fig. 2, A and C), indicating that Hsp72 does not have a general effect on UV-induced JNK-signaling pathway but specifically suppresses JNK activity.

It is noteworthy that UV-triggered activation of JNK was also suppressed when elevation of Hsp72 levels was achieved by a physiologically more relevant method, i.e. by heat shock pretreatment of cells. Fibroblasts were exposed to heat shock at 45 °C for 45 min followed by recovery for 18 h, which caused strong induction of Hsp72 (Fig. 2D). In these cells JNK activation by UV irradiation was strongly suppressed as compared with nonpretreated control cells (Fig. 3, A and B). Heat shock pretreatment did not influence the level of JNK expression (data not shown). Similarly, endogenous Hsp72 induced by mild heat shock pretreatment suppressed activation of JNK by IL-1 (Fig. 3, C and D). These data further indicate that Hsp72 at elevated levels is able to inhibit activation of JNK by stimuli that do not cause protein damage.

Heat shock at 45 °C for 45 min, neither UV-C nor IL-1 caused induction of Hsps (Fig. 2D). Therefore, the latter stimuli appear not to produce significant protein damage.

To test whether upon UV stimulation of cells Hsp72 affects JNK specifically or causes a general suppression of the JNK-signaling pathway, we investigated the effects of adenoviral expression of Hsp72 on activation of SEK1, an immediate upstream kinase in the JNK pathway. JNK can also be activated by another member of SEK1 family, MKK7 (25). However, SEK1 is critical for UV-induced activation of JNK, since UV-triggered induction of JNK is dramatically reduced in embryonic fibroblasts and stem cells derived from SEK1 knock-out mice (26, 27). Control and Hsp72-expressing cells exposed to UV irradiation were analyzed by immunoblotting of cellular lysates with anti-phospho-SEK1 antibody specific for the active
Hsp72, Cdc42V12 activated JNK1-HA less than 2-fold (Fig. 4B). A similar approach was used to investigate whether Hsp72 can inhibit activation of JNK by constitutively active MEKK1 (see scheme in Fig. 7). COS-7 cells were transfected with N-terminally truncated MEKK1 (MEKK1Δ) along with JNK1-HA. Expression of MEKK1Δ resulted in 7–10-fold activation of JNK1-HA in control cells. However, in cells cotransfected with Hsp72-expressing plasmid, this activation was strongly inhibited (Fig. 4B). The level of JNK1-HA was not affected by expression of Hsp72 (Fig. 4B). From these experiments we conclude that Hsp72 can inhibit activation of JNK by the active upstream elements of the JNK-signaling pathway.

To test whether elevated expression of Hsp72 specifically affects JNK or causes a general suppression of the JNK-activating pathway, we investigated the effects of Hsp72 on activation of SEK1 triggered by constitutively active MEKK1. For these experiments we employed a GST fusion of a kinase-dead mutant version of SEK1 (SEK1K/R). SEK1K/R can be efficiently phosphorylated by MEKK1 at the proper site but cannot either autophosphorylate or phosphorylate JNK (15). Therefore, in this experimental system phosphorylation of SEK1K/R, which directly reflects its activity, depends entirely on the upstream kinase MEKK1 and cannot be influenced by potential feedback activation by JNK.

When GST-SEK1K/R was transfected into COS-7 cells it remained unphosphorylated and was not detected by anti-phospho-SEK1 antibody. However, when MEKK1Δ was coexpressed along with GST-SEK1K/R, the latter became phosphorylated (Fig. 4C). Transiently expressed Hsp72 in these cells at a level sufficient to inhibit MEKK1Δ-induced activation of JNK1-HA did not suppress MEKK1Δ-induced activation of SEK1K/R (Fig. 4, compare B and C). Expression of GST-SEK1K/R was not affected by the presence of Hsp72 plasmid, as seen on the immunoblots of the same membrane with anti-GST antibody (Fig. 4C). Similar results were obtained when cells were transfected with a plasmid-expressing GST fused to wild type SEK1 (not shown). Therefore, neither in fibroblasts nor in COS-7 cells did Hsp72 cause general suppression of the JNK-signaling pathway but rather specifically suppressed the activation of JNK.

**Hsp72 Suppresses Activation of JNK by Phosphatase Inhibitors**—As mentioned in the Introduction, JNK can be activated either via the activation of upstream kinase cascade or through the inhibition of JNK dephosphorylation. All the stimuli tested above, including UV irradiation, IL-1, Cdc42, and MEKK1 stimulate JNK via the activation of upstream kinases (4, 29). In contrast, heat shock does not activate these kinases but instead blocks JNK dephosphorylation (17). When JNK dephosphorylation is blocked, the background activity of SEK1 becomes sufficient to account for the activation of JNK (see scheme in Fig. 7) (17).

To test if Hsp72 can suppress JNK activation triggered by inhibition of JNK dephosphorylation, we employed a combination of two phosphatase inhibitors, orthovanadate and calyculin A, that previously were found to cause rapid inhibition of JNK dephosphorylation in unstimulated cells (17). Control and Hsp72-expressing cells were incubated with the phosphatase inhibitors, and the levels of phosphorylation of two major JNK isoforms, JNK1 and JNK2, were assayed by immunoblotting of cell lysates with anti-phospho-JNK antibody. Since only phosphorylated JNK is active, the level of JNK phosphorylation directly reflects its activity (see Introduction). Treatment of control fibroblasts with phosphatase inhibitors for 5 min results in strong activation of JNK, comparable with the activation of JNK achieved by UV irradiation (Fig. 5). The effect of phosphatase inhibitors was likely due to inhibition of JNK dephosphorylation rather than activation of the upstream kinase cascade, since SEK1 remained inactive at this time point (Fig. 5; activation of SEK1 by UV irradiation is given for comparison). In contrast to the control cells, in Hsp72-expressing cells activation of JNK by phosphatase inhibitors was markedly suppressed (Fig. 5, top panel, lanes 3 and 4). These
data indicate that JNK activation triggered either by stimulation of the upstream kinase cascade or by inhibition of JNK dephosphorylation can be efficiently suppressed by Hsp72.

Hsp72 Mutant Lacking the ATPase Domain Is Able to Suppress JNK Activation—Since Hsp72 can suppress activation of JNK by stimuli that do not cause protein damage, we next tested if the protein refolding activity of Hsp72 is necessary for JNK suppression. For these experiments we utilized the C-terminal fragment of Hsp72 (CTF) which contains the peptide binding domain but lacks the ATPase activity of the intact molecule. CTF can bind to unfolded proteins but cannot dissociate from them and, therefore, cannot facilitate their refolding.

As in the experiments described above, COS-7 cells were transiently transfected with the constitutively active mutant form of Cdc42 (Cdc42V12) along with JNK1-HA and were cotransfected with either empty vector or vector-expressing CTF. Since CTF is not recognized by the monoclonal anti-Hsp72 antibody (SPA820) (Fig. 6A), Cdc42V12 activated JNK1-HA less than 2-fold in cells cotransfected with plasmid-expressing CTF (9.5 μg) (marked “+” in the figure) or with empty vector (9.5 μg) (marked “−” in the figure). Forty eight hours later cells were lysed, and levels of GST-SEK1/R phosphorylation were assayed by immunoblotting of cellular lysates with anti-phospho-SEK1 antibody. Lower panel, levels of GST-SEK1/R in precipitates from the upper panel were assayed by immunoblotting of the membrane with anti-GST antibody.

FIG. 7. Scheme of two pathways of JNK activation by different stresses. JNK can be activated either through upstream kinase cascade or via inhibition of JNK phosphatase.
along with JNK1-β and cotransfected with either empty vec-
tor or vector-expressing CTF. In cells that did not express CTF,
along with JNK1-HA and cotransfected with either empty vec-
tor or vector-expressing CTF. In cells that did not express CTF,
the ATPase activity of Hsp72 is dispensable for its
inhibitory effects on JNK, and therefore the protein refolding activity is unnecessary for this function of Hsp72.

**DISCUSSION**

Previously we have demonstrated that the heat shock protein
Hsp72 at elevated levels suppresses activation of the stress kinase JNK by heat shock and ethanol, treatments known to
cause protein damage (18). There are two possible interpreta-
tions of these data: first, Hsp72 protects or promotes refolding of a negative regulator of the JNK-signaling pathway damaged by stress, thus reducing the JNK-activating signal; second, that Hsp72 plays a regulatory role in the JNK signaling. The critical test of these alternatives was to study whether Hsp72 is able to suppress JNK activation by stimuli that do not cause protein damage. Here we have observed that elevated levels of either recombiant Hsp72 or endogenous Hsp72 after mild heat pretreatment of cells suppress JNK activation by IL-1 or UV irradiation. Hsp72 also suppresses activation of JNK by constitutively active forms of either Cdc42 or MEKK1. Moreover, Hsp72 strongly reduces activation of JNK induced by phosphatase inhibitors. Since neither of these stimuli cause significant protein damage, the function of Hsp72 in JNK suppression seems to be unrelated to its role in the protection of proteins from damage.

We also demonstrate here that deletion of the ATPase
domain of Hsp72 does not prevent it from suppressing JNK when induced by constitutively active mutant forms of either MEKK1 or Cdc42. These data indicate that ATPase activity of Hsp72, which is critical for its role in protein refolding, is dispensable for Hsp72-mediated inhibition of JNK. Furthermore, since the peptide binding domain is intact in CTF, this fragment is able to form complexes with target proteins suggesting that association of Hsp72 with a component of the JNK-signaling pathway is critical for JNK regulation, whereas ATP-dependent dissociation of such complex is unnecessary. Taken together these data suggest that Hsp72 is implicated in the regulation of JNK signaling. Analogously, the close homologue of Hsp72, Hsc73, plays a purely regulatory role in the repression of the heat shock transcription factor HSF1 (30, 31) or the eIF2 kinase (32).

How does Hsp72 regulate JNK signaling? In related work we have found that activation of JNK by protein-damaging stresses proceeds via inhibition of JNK phosphatase (17). We also demonstrated that Hsp72 suppresses JNK activation induced by protein-damaging agents via acceleration of JNK dephosphorylation (see Fig. 7) (17). The fact that Hsp72, while suppressing activation of JNK induced by either UV irradiation or MEKK1α, fails to suppress activation of SEK1 by these agents (Figs. 2B and 4C) suggests that Hsp72 works in the pathway downstream of SEK1. This is consistent with a role of Hsp72 in up-regulation of JNK dephosphorylation. (Technically, it was not feasible to test this possibility in direct experi-
ments since the rate of JNK dephosphorylation in UV-irradiated and IL-1-treated cells is high and beyond the resolution of the experiment.) As with Hsp72, it is likely that CTF reduces JNK activation by Cdc42/β and MEKK1α by accelerating JNK phosphatase.

Data presented here indicate that expression of Hsp72 controls activation of JNK by a wide variety of stimuli that do not depend on protein damage. Since JNK is critical for apoptosis induced by certain DNA-damaging agents and UV irradiation (6, 8, 9), Hsp7-mediated suppression of JNK activation may explain the long known phenomenon of the resistance of cells to various
drugs, which is acquired after mild heat pretreatment of cells (33–36). Indeed, exposure to mild heat shock protects cells from
adriamycin (doxorubicin) (33) and etoposide (35), as well as from UV irradiation (37). Based on our new findings, we suggest that Hsp72 induced by mild heat shock suppresses activation of JNK by these agents, which ultimately leads to inhibition of apoptosis and enhances cell survival (although it should be noted that Hsp72 may affect apoptotic pathway downstream of JNK as well (38)). Therefore, our findings provide evidence for a general role of Hsp72 in control of stress kinases.