Hsp70 promotes TNF-mediated apoptosis by binding IKKγ and impairing NF-κB survival signaling

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The major heat shock protein, Hsp70, can protect against cell death by directly interfering with mitochondrial apoptosis pathways. However, Hsp70 also sensitizes cells to certain apoptotic stimuli like TNF. Little is known about how Hsp70 enhances apoptosis. We demonstrate here that Hsp70 promotes TNF killing by specifically binding the coiled-coil domain of IκB kinase γ (IKKγ) to inhibit IKK activity and consequently inhibit NF-κB-dependent antiapoptotic gene induction. An IKKγ mutant, which interacts with Hsp70, competitively inhibits the Hsp70–IKKγ interaction and relieves heat-mediated NF-κB suppression. Depletion of Hsp70 expression with RNA interference rescues TNF-mediated cell death. Although TNF may or may not be sufficient to trigger apoptosis on its own, TNF-triggered apoptosis was initiated or made worse when Hsp70 expression increased to high levels to disrupt NF-κB signaling. These results provide significant novel insights into the molecular mechanism for the pro-apoptotic behavior of Hsp70 in death-receptor-mediated cell death.

[Keywords: Hsp70; NF-κB; IKKγ; TNF; apoptosis; death receptor signaling]

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Even though heat shock proteins are known to inhibit various types of apoptosis, some studies show that heat or elevated heat shock protein 70 (Hsp70) also potentiates cell death following specific stimuli. For instance, overexpression of HSFI (heat shock transcription factor 1, one transcription factor that controls the heat shock response) enhances Fas-induced cell apoptosis [Xia et al. 2000]. Elevated Hsp70 promotes TCR/CD3- and Fas/Apo-1/CD95-mediated Jurkat T-cell apoptosis [Liossis et al. 1997]. Heat increases cell death following some chemotherapeutics and radiation [Dewey 1984], and heat shock also sensitizes AML cells and endothelial cells to apoptosis [Chant et al. 1996; DeMeester et al. 1998]. Hsp70 inhibits cellular proliferation [Maheswaran et al. 1998]. Moreover, it is also well established that heat shock or elevated Hsp70 alters the regulation of signaling cascades and transcription factors and potently sensitizes tumors to radiation [Curry et al. 1999]. These results imply that heat or Hsp70 could disable a cell survival signal under the appropriate circumstances.

NF-κB is sequestered in the cytoplasm in an inactive complex with IκB proteins [Huxford et al. 1998; Malek et al. 2001]. Activation of the NF-κB-mediated signal is initiated through degradation of phosphorylated IκB. Various stimuli activate the IκB kinase [IKK] complex, which in turn phosphorylates IκB, leading to NF-κB activation (Chen et al. 1995; Zandi et al. 1997). NF-κB is critical for survival of most cells through the induction of antiprototic genes [Beg et al. 1995; Beg and Baltimore 1996; Van Antwerp et al. 1996; Wang et al. 1998; Zong et al. 1999; Rudolph et al. 2000]. IKK plays a central role in mediating NF-κB activation. IKK is composed of two catalytic subunits, IKKα and IKKβ, which can directly phosphorylate IκB. IKKγ (also called NEMO) is an absolutely essential regulatory component of the IKK complex that is necessary for NF-κB activation [Rothwarf et al. 1998; Yamaoka et al. 1998; Makris et al. 2002].

A number of studies have also shown that heat shock or elevated Hsp70 suppresses NF-κB activity [Feinstein et al. 1997; Guzhova et al. 1997; Curry et al. 1999; Andrees et al. 2002; Malhotra and Wong 2002]. Although these studies imply the possibility that Hsp70 impairs NF-κB signaling, the exact molecular basis of the Hsp70 and NF-κB interaction is still enigmatic.

This study therefore examined the mechanism by which Hsp70 interacts with NF-κB and might promote apoptosis. To do this, we studied several cell lines [Cos-1, Hela, 293 cells] that do not apparently undergo apo-
Hsp70 enhances apoptosis by blocking NF-κB signaling

Hsp70 did not directly interfere with the function of the NF-κB activation domain using a cotransfection assay with plasmid encoding Gal4 [DNA-binding domain]–p65 (activation domain) chimeric protein, a Gal-4 reporter, and Hsp70 construct (data not shown). Next, to analyze the effect of Hsp70 on NF-κB DNA-binding activity, we performed a gel mobility shift assay using a p65 probe and nuclear extracts isolated from Cos-1 cells. Heat, Hsp70, IκBαDN, and HSF1 inhibited NF-κB DNA binding (Fig. 1E, lanes 1–8). The effects of HSF1 were not a direct effect on the NF-κB activity because Hsp70 antisense [Hsp70AS] blocked the effect, showing that it is HSF1 induction of Hsp70 that inhibits NF-κB DNA binding (Fig. 1E, lane 8). Cellular localization data showed that Hsp70 inhibited TNF-induced translocation of p65 from the cytoplasm to the nucleus (Fig. 1F).

To test whether Hsp70 and HSF1 directly affect the NF-κB DNA-binding activity, we translated p65, Hsp70, and HSF1 proteins in vitro and performed the aforementioned gel mobility shift assays. Hsp70 and HSF1 failed to directly influence the NF-κB DNA binding (Fig. 1G). These negative data are shown because they help rule out the possibility that Hsp70 or HSF1 directly regulates NF-κB DNA-binding activity. In order to further rule out the possibility that Hsp70 directly interacts with NF-κB (p65), we constructed NLS/p65 plasmids with forced p65 nuclear localization that was independent of IκBα degradation. Cos-1 cells were cotransfected with NLS/p65, NF-κB reporter, and Hsp70 constructs. NF-κB reporter activity analysis showed that Hsp70 did not suppress NF-κB activity (data not shown). This confirmed the earlier findings that Hsp70 did not directly interact with NF-κB (p65).

Hsp70 inhibits TNF-induced phosphorylation of IκBα

Because Hsp70 inhibited NF-κB activity without directly binding NF-κB, this indicated that Hsp70 impaired IκBα signaling upstream of NF-κB (p65). Because activation of NF-κB requires phosphorylation of IκB, we examined the effects of Hsp70 on the phosphorylation of IκB using specific antibodies to examine phosphorylated and unphosphorylated IκBα proteins. The results showed that Hsp70 inhibited TNF-induced IκBα phosphorylation (Fig. 2A).

These data suggested either that Hsp70 directly inhibited κB activity, or that Hsp70 directly bound IκBα to mask its phosphorylation site, or that Hsp70 accelerated IκBα dephosphorylation. In order to differentiate between these possibilities, several experiments were performed. First, pull-down data, immunoprecipitation data, and two-hybrid experiments showed that Hsp70 did not interact with IκBα (see data below). Second, Hsp70- and Hsp70C-inducible HeLa cells were transiently transfected with Flag-IKKβ and Flag-IKKβ. The effect of Hsp70 on IKK activity was assayed using an in vitro kinase assay with exogenous GST-IκBα (1–56) as the substrate. Hsp70 reduced IKK activity in a dose-dependent manner (Fig. 2B). Neither Hsp70 nor Hsp70C altered IKKβ expression (Fig. 2B).

Results

Hsp70 inhibits NF-κB activity

To determine whether Hsp70 itself specifically suppressed NF-κB activity, we cotransfected Cos-1 cells with an NF-κB reporter and the indicated constructs. In this transfection assay, Hsp70 and IκBαDN [IκBα dominant negative] suppressed NF-κB reporter gene activity, whereas Hsp70C [C terminus of Hsp70] and Hsp70N [N terminus of Hsp70] failed to do so (Fig. 1A). HSF1 also inhibited NF-κB activity. However, this inhibition was abrogated by cotransfection with Hsp70AS [antisense Hsp70], indicating that HSF1 did not directly affect NF-κB activity (Fig. 1A). Hsp90 did not affect NF-κB activity in resting cells but enhanced NF-κB activity when cells were treated with TNF (Fig. 1A).

To demonstrate the specificity of the Hsp70 inhibition, we did similar experiments with AP-1- and HSF-dependent reporters. It was shown that Hsp70 did not significantly affect activation of AP-1, whereas both Hsp70 and Hsp70C efficiently inhibited HSF activity (Fig. 1B). This is consistent with findings that Hsp70 can inhibit HSF1 transcriptional activity (Abravaya et al. 1992; Shi et al. 1998). An additional control experiment showed that Hsp70 had similar effects on the repositioning of β-gal and luciferase, as measured by their enzymatic activities [data not shown]. Therefore, in all of the transfection assays shown, the elevated Hsp70 did not artificially decrease or increase the luciferase readings when normalized to β-gal.

To determine whether physiologically elevated levels of Hsp70 inhibit NF-κB activity, we established Hsp70-inducible cell lines using the nonintegrating plasmid pMEP4 under the control of a Zn2+-regulated metallothionein promoter. Hsp70 dose-dependently inhibited NF-κB activity (Fig. 1C). The modest reduction of NF-κB activity in Hsp70C-expressing cells (Fig. 1C, lanes 2–4) likely occurred because of Zn2+-mediated induction of endogenous Hsp70 (Fig. 1C, D). Cells treated with TNF alone, in the absence of ZnCl2, had a relative luciferase activity for NFκB-luc of 100 ± 8 [see results following], which is similar to the relative luciferase activity levels for the cells stably expressing His-Hsp70C in the presence of ZnCl2 (Fig. 1C, lanes 2–4) and for His-Hsp70 cells treated with low levels of ZnCl2 [Fig. 1C, lane 6].
Hsp70 directly interacts with IKKγ

On the basis of these observations, we considered whether Hsp70 directly interacted with one or more components of the IKK complex and inhibited IKK activity. To determine which molecule(s) of the IKK complex might be the primary target of Hsp70, we searched for a direct interaction between the three IKKs and Hsp70 using a beads pull-down assay. 35S-labeled IKKγ/H9253 was retained on Hsp70-, but not on Hsp70C- conjugated beads. IKKα and IKKβ did not associate with Hsp70 or with Hsp70C (Fig. 3A).

To map which region within IKKγ was responsible for this interaction, we constructed various deletions of IKKγ [Fig. 3B]. The in vitro beads pull-down assay showed that only the IKKγ and IKKγC1, which contained the coiled-coil motif, interacted with Hsp70 [Fig. 3C], suggesting that Hsp70 bound to the coiled-coil motif of IKKγ.
To further confirm that Hsp70 associated with IKKγ within cells, we performed a two-hybrid assay in cultured mammalian cells. Hsp70 and Hsp70C CDNAs were in frame cloned into the pM vector containing the Gal-4 DNA-binding domain. Similarly, the IKKγ and its mutants, and IKKα, IKKβ, and IκBα CDNAs were in frame inserted into pVP16 plasmid containing the activation domain. The resulting constructs were cotransfected into Cos-1 cells with a Gal-4 reporter gene. The results confirmed the in vitro binding data showing Hsp70 interaction with IKKγ and IKKγC1 but not with IκBα, IKKα, or IKKβ (Fig. 3D). Hsp70C did not associate with any of the molecules tested (Fig. 3D).

To determine whether endogenous Hsp70 interacted with IKKγ at physiologically relevant levels, we exposed cells to heat and TNF, either with GFPsiRNA (control, Fig. 3E, lanes 1–3) or with Hsp70siRNA (Fig. 3E, lanes 4–6). The cells were lysed and immunoprecipitated with an anti-Hsp70 antibody. The amounts of IKKγ protein in the anti-Hsp70 immunoprecipitates were assessed with anti-IKKγ antibody (Fig. 3E). Portions of the total cell extracts were immunoblotted with β-actin antibody as a control for lane loading or for siRNA specificity. The results confirmed that detectable amounts of endogenous IKKγ were present in the anti-Hsp70 immunoprecipitates [Fig. 3E, second gel from top] but not in the antiserum control [Fig. 3E, lane 1]. In addition, IKKγ specifically associated with endogenous Hsp70, as the Hsp70–IKKγ interaction was markedly decreased in Hsp70siRNA transfectants [Fig. 3E, second gel from top, lanes 4–6]. IKKγ activity was analyzed next using an anti-phospho-IκBα [P-IκBα] antibody. As expected, elimination of Hsp70 by RNAi rescued heat-mediated inhibition of IκBα phosphorylation [Fig. 3E, top gel, lanes 3–6]. Conversely, using a similar approach, the endogenous Hsp70 could also be detected in the anti-IKKγ immunoprecipitates [Fig. 3E, second gel from top, lanes 2–4] but not in the antiserum control [Fig. 3F, lane 1]. IKKγ activity, as assessed by P-IκBα levels, decreased [Fig. 3E, top gel] with decreasing levels of IKKγ [Fig. 3F, third gel from top].

Hsp70 inhibits the formation of the IKK complex
To define the interactions of the components of IKK and the effects of Hsp70 on the IKK complex in detail, we used the mammalian two-hybrid system to determine whether Hsp70 not only bound IKKγ, but also disrupted interactions between the three IKKs. IKKα, IKKβ, and IKKγ were immunoblotted with an anti-His antibody (top gels). An in vitro kinase assay was performed using GST–IκBα (1–56) as substrate (middle gels). IKKβ protein levels were determined with an anti-Flag antibody (lower gels).

To define the effects of Hsp70 on IKK complex assembly and IKK activity, we transfected 293 cells with His-Hsp70 or His-Hsp70C. The extracts were immunoprecipitated with an anti-IKKγ antibody. The amounts of IKKα and IKKβ proteins in the anti-IKKγ immunoprecipitates were detected with anti-IKKα/β antibody [Fig. 4B, second gel], and in vitro kinase assays were performed on the immunoprecipitated IKK complexes using exogenous GST–IκBα fusion protein as substrate. Transfection with Hsp70 (third gel from the top) abolished the robust activation of IKK in response to TNF stimulation [Fig. 4B, uppermost gel] and decreased the amounts of IKKα and IKKβ proteins in the anti-IKKγ immunoprecipitates (Fig. 4B, second gel from top). IKKα/β expressions were not changed (Fig. 4B, fourth gel from top).

These data suggested that Hsp70 might inhibit the assembly of the IKK complex. To test this possibility, we transiently transfected Hsp70- and Hsp70C-inducible Hela cells with Flag-IKKα, Flag-IKKβ, and HA-IKKγ constructs for 48 h. Following challenge with TNF, cells were extracted and subjected to gel filtration on a Superose 6 column. Each column fraction was immunoblotted with anti-Flag [IKKα/β], anti-HA [IKKγ], and anti-His [Hsp70, Hsp70C] antibodies. IKKγ activity was measured using an in vitro kinase assay. Hsp70 reduced the size of the IKK complex and markedly increased the amounts of IKKγ in the lower molecular weight fractions (fourth row) in comparison with those from Hsp70C (third row) transfected cells (Fig. 4C). Both the HA-IKKγ [fourth row] and His-Hsp70 [sixth row] were detected in the same column fractions migrating at ~120 kDa, a finding consistent with stable Hsp70–IKKγ heterocomplexes. As shown earlier, Hsp70 inhibited IKK activity [Fig. 4C, bottom two rows].

Because these results suggested that the Hsp70–IKKγ interaction was critically involved in regulating NF-κB signaling, we confirmed whether disruption of IKKγ–Hsp70 interaction would be sufficient to overcome
Hsp70-mediated NF-κB suppression. Because Hsp70 directly bound both IKKγ and IKKγC1, but not IKKγC2 [Fig. 3C,D], we used IKKγC1 to selectively disrupt the interaction between IKKγ and Hsp70 by competing for binding to Hsp70. Therefore, 293 cells were transfected with HA-IKKγ and increasing amounts of HA-IKKγC1 or HA-IKKγC2. Cells were exposed to TNF to activate NF-κB and exposed to heat shock (45°C) for 30 min to induce endogenous Hsp70, and cell extracts were immunoprecipitated with anti-Hsp70 antibody. Appreciable amounts of HA-IKKγ protein could be detected with anti-HA antibody in the anti-Hsp70 immunoprecipitates [Fig. 4D, top gel, lanes 3–5] but not in the antiserum control [Fig. 4D, top gel, lane 1]. Another portion of these cells was transfected with an NF-κB reporter gene for NF-κB activity analysis. Immunoprecipitation showed that Hsp70 interacted with IKKγ and that IKKγC1 displaced IKKγ from Hsp70 immunocomplex [Fig. 4D, top gel, lanes 3–5,7–9]. IKKγC1-mediated IKKγ release from the Hsp70–IKKγ complex was accompanied by gradual restoration of NF-κB activity [Fig. 4D, upper panel, lanes 7–9], whereas IKKγC2 had no such effect [Fig. 4D, upper panel, lanes 3–5].

Hsp70 precludes IKKγ oligomerization, which is required for NF-κB activation

These data showed that IKKγ formed homo-oligomers [Fig. 4A] and that the coiled-coil motif of IKKγ was the Hsp70-binding site [Fig. 3B,C], suggesting that the coiled-coil region serves as an effector domain to regulate the IKK complex via its oligomerization. If this conclusion is correct, the coiled-coil deletion mutant of IKKγ should not self-associate and should suppress NF-κB signaling. We tested this by transfecting Cos-1 cells with IKKγC [the coiled-coil deletion mutant of IKKγ, Fig. 3B] and either Gal4- or NF-κB-dependent reporters. The IKKγC failed to interact with itself or IKKα and IKKβ, even on TNF treatment [Fig. 5A]. In addition, the IKKγCC, like Hsp70, inhibited TNF-mediated NF-κB activation [Fig. 5B]. These effects of Hsp70 and IKKγC on suppressing TNF-mediated NF-κB activation are specific
because our previous data showed that neither Hsp70C nor Hsp70N affected NF-κB activity (Fig. 1A). These data suggested that IKK oligomerization via the coiled-coil motif was indispensable for IKK activity.

To further examine the effects of Hsp70 on IKK oligomerization and its regulation of IKK activity, we transfected 293 cells with HA-IKK/oligomerization and its regulation of IKK activity, we transfected 293 cells with HA-IKK/CC or HA-IKK/C. Some of the cells were cotransfected with Hsp70 or Hsp70C. All cells were metabolically labeled with 35S-methionine and some were then briefly exposed to TNF. In vivo chemical cross-linking experiments in 293 cell extracts were performed using the homobifunctional cross-linker ethyleneglycol-bis-succinimidylsuccinate (EGS), then were immunoprecipitated with anti-HA antibody or with anti-IKK/β antibody (for IKK-depleted extracts). Some extracts were immunodepleted with an anti-IKK antibody prior to immunoprecipitation. To analyze the oligomeric state of IKK/β, we analyzed the cross-linked, immunoprecipitated complexes by SDS-PAGE. We readily detected the different IKK/β oligomeric states with masses of >250 kDa (Fig. 5C, lane 3), which was not the case for IKK/CC transfected cells (Fig. 5C; lanes 1,2).

The IKK/β multimers containing the IKK/β/κα/β complexes may be necessary for basal NF-κB activity (Fig. 5C, lane 3). TNF treatment resulted in formation of the high-molecular-weight IKK complex (Fig. 5C, lanes 4,7), whereas immunodepletion of IKK/β and transfection with Hsp70 markedly decreased the amount of the high-molecular-weight IKK complex (Fig. 5C, lanes 5,6). To determine whether the catalytic activity of the IKK complex required IKK/β oligomerization, we retrieved IKK complexes from the transfected cells by immunoprecipitation with anti-HA/IKK/β antibody and examined them for in vitro kinase activity.
Figure 5. Hsp70 inhibits the IKKγ oligomerization required for NF-κB activation. [A] Two-hybrid experiment for protein interactions. Cos-1 cells were transiently transfected with a Gal-4-dependent reporter and the indicated constructs and then subjected to luciferase analysis 24 h later. [B] IKKγCC and Hsp70 inhibit TNF-induced (25 ng/mL) NF-κB activation. Cos-1 cells were transfected with NF-κB-Luc together with either IKKγCC or Hsp70 and then subjected to luciferase analysis 24 h later. [C] IKKγ formed oligomers. 293 cells expressing HA-IKKγ (lanes 3–7) or HA-IKKγCC (lanes 1,2) were labeled with 35S-methionine. Some of the cells were transfected with Hsp70 (lane 6) or Hsp70C (lane 7) expression vectors. Cells were treated with vehicle or TNF (25 ng/mL). Cells were lysed and the extracts were incubated with cross-linker (EGS). After cross-linking, one batch had IKKγ immunodepleted using IKKγ antibody (lane 5). IKKγ was immunoprecipitated with HA antibody [lanes 1–4,6,7], and the IKKγ immunodepleted extracts were immunoprecipitated with an anti-IKKα/β antibody (lane 5). The pellets were washed and analyzed by SDS-PAGE. IKK activity [KA] in the immunoprecipitates was determined with GST–IkBα as substrate (bottom gel). [D] Hsp70 inhibits the IKKγ oligomerization in vitro. 35S-labeled IKKγ and IKKγCC were incubated with or without purified Hsp70 and the cross-linking agent (EGS). The proteins were analyzed by SDS-PAGE. [E] IKKγ or IKK complexes can be visualized in vivo and are inhibited by Hsp70 or heat. GFP–IKKγ, GFP–IKKγCC, or RFP–Hsp70 were expressed alone (panel a) or together (panels b–c) in HeLa cells. Nontransfected cells were exposed either to vehicle (panel d), TNF (25 ng/mL for 15 min, panel e), or TNF (25 ng/mL for 15 min) plus heat (30 min at 45°C, panel f). They were then fixed and double immunostained with anti-IKKγ and anti-IKKα/β antibodies, and fluorescent signals were visualized by confocal microscopy.
Only IKKγ-transfected cells that were treated with TNF had high IKK activity [Fig. 5C, lanes 4,7]. IKK activity was greatly decreased in TNF-treated cells by IKKγ CC transfection [Fig. 5C, lane 2], by Hsp70 cotransfection [Fig. 5C, lane 6], and following IKKγ depletion [Fig. 5C, lane 5]. These results indicate that inducible IKK activity is critically dependent on IKKγ or its oligomerization. These in vivo data also suggest that overexpression of Hsp70 but not Hsp70C blocks formation of the IKK complex and favors formation of Hsp70–IKKγ heterodimers [Fig. 5C, lane 6].

Therefore, we next determined whether Hsp70 would block IKKγ oligomerization in vitro and whether IKKγ oligomerization was dependent on any other proteins. 

To further confirm the effects of Hsp70 on IKKγ or IKK complex in living cells, we transfected GFP/IKKγ (GFP, green fluorescent protein), GFP/IKK-CC, and RFP/IKK γ (RFP, red fluorescent protein) into Hela cells as reporters and examined their subcellular localization using confocal microscopy. Expression of GFP/IKKγ demonstrated multifocal, punctate regions of staining. They could be macromolecular foci composed of either IKKγ oligomeric complexes and/or complexes of IKKγ with other proteins. This contrasted with IKKγ-CC expression, which showed diffuse, uniform fluorescence [Fig. 5E, panel a]. These findings were observed in >95% of the cells in which GFP/IKKγ or GFP/IKK-CC was expressed. Cotransfection of GFP/IKKγ and RFP/Hsp70 resulted in a dramatic redistribution of IKKγ from discrete macromolecular foci to uniform fluorescence [Fig. 5E, panel c]; the findings being observed in >60% of cells in which both GFP/IKKγ and RFP/Hsp70 were coexpressed in some cells, the levels of Hsp70 expression may be not sufficient for the redistribution of IKKγ). In nontransfected cells, double labeling with antibodies to IKKγ and IKKα/β again showed macromolecular foci in TNF-treated cells [Fig. 5E, panel c], whereas there were no IKKγ/IKK foci in control [no TNF treatment, Fig. 5E, panel d] or heat-shocked [Fig. 5E, panel f] cells [Fig. 5E]—the findings again being observed in >95% of cells. These data represent a clear in vivo demonstration of the ability of Hsp70 to inhibit formation of these IKKγ immunostained foci. The data are consistent with Hsp70 interfering with the formation of IKKγ oligomers [dimers, trimers, tetramers], and/or with Hsp70 interfering with the association of IKKγ with other proteins that make up the IKK macromolecular complex.

Hsp70 enhances apoptosis by blocking NF-kB signaling

Because Hsp70 suppression of NF-kB activity could adversely affect cell survival, we investigated the effects of Hsp70 on cell survival following treatment with several stressors. We compared the apoptotic behavior of control Hela cells to IkBαDN [IkBαDN-dominant negative used to block NF-kB activation] and Hsp70 stable Hela cells. In many of the experiments, the cells were exposed to TNF for 4 h and/or heating to 45°C for 30 min. Cell death was detected by examination of the nuclear morphology using Hoechst staining. Normal cell survival was observed for cells with vector alone, vector + TNF, Hsp70C + TNF, or Hsp70, or when heated alone [Fig. 6A]. Cell death, manifested by nuclei with intensely condensed and occasionally fragmented morphology, was observed with cells treated with TNF plus any of the following three treatments: overexpressing IkBαDN, overexpressing Hsp70, or heating [Fig. 6A].

Quantitative evaluation of Hela cell survival showed that treatment of Hela cells with LPS, PMA, STS, and serum deprivation increased to 60%, whereas TNF treatment plus Hsp70 decreased survival from 95% [Fig. 6B, left panel]. Following expression of Hsp70, however, cell survival following LPS, PMA, STS, and serum deprivation increased to 60%, whereas TNF treatment plus Hsp70 decreased survival from 95% [Fig. 6B, left panel] to ~50% [Fig. 6B, middle panel]. Expression of Hsp70C combined with TNF treatment was associated with nearly normal 95% cell survival [Fig. 6B, right panel], similar to the cell survival with TNF treatment alone [Fig. 6B, left panel].
Figure 6. Effects of Hsp70 and NF-κB on cell survival. (A) Hsp70-, Hsp70C-, and IkBaDN-inducible Hela cells were treated with ZnCl₂ (100 µM) for 4 h. Sixteen hours later, the cells were treated with vehicle or TNF (50 ng/mL) for 4 h. Nuclei were stained with Hoechst. (B) Hsp70- or Hsp70C-inducible Hela cells had Hsp70 [middle panel] or Hsp70C [right panel] induced with ZnCl₂ (100 µM) for 4 h. Sixteen hours later, all cells were treated with the indicated agents. Cell viability was assessed 24 h later using MTT. (C) IkBaDN-, Hsp70-, or Hsp70C-inducible Hela cells, or cells with empty vector [pMEP4], were treated with increasing concentrations of ZnCl₂ for 4 h. Sixteen hours later, cells were treated with vehicle or TNF (50 ng/mL). Cell viability was assessed 24 h later using MTT. (D) NF-κB rescues Hsp70–TNF-induced cell apoptosis. Hsp70-inducible Hela cells were transiently transfected with an NF-κB/p65 expression vector. Twenty-four hours later, the cells were treated with ZnCl₂ (100 µM) for 4 h to induce Hsp70. TNF (50 ng/mL) or vehicle was then added and cell viability assessed 24 h later using MTT. (E) IKKγ overcomes TNF–Hsp70-induced cell apoptosis. Hsp70-inducible Hela cells were transiently transfected with IKKγ or IKKγCC or empty vector. Twenty-four hours later, the cells were treated with vehicle or with ZnCl₂ (100 µM) for 4 h to induce Hsp70. Sixteen hours later, TNF (50 ng/mL) or vehicle was added and cell viability assessed 24 h after that using MTT. (F) IkBaDN-, Hsp70-, or Hsp70C-inducible Hela cells were treated with ZnCl₂ (100 µM) for 4 h. Sixteen hours later, cells were then treated with vehicle or TNF [50 ng/mL]. Twenty-four hours later, Western blots were performed on cell extracts with antibodies to PARP, IkBa, and β-actin. NF-κB activity was assessed in cells under the same conditions using a luciferase assay [bottom panel]. (G) The effects of Hsp70–IKKγ interactions on IKK activity and cell survival. HA-IKKγ or Hsp70 siRNA [lanes 8,9] or GFPsiRNA [lanes 1–4,6,7] were transiently transfected into 293 cells. After 48 h, the cells were treated either with vehicle, with TNF (50 ng/mL), and/or with heat (30 min at 45°C). Cells were lysed immediately, and the lysates immunoblotted with anti-phospho-IκBα (top gel), anti-Hsp70 [second gel down], anti-IKKγ [third gel down], and β-actin antibodies. The survival of similarly treated cells was determined 24 h later using MTT. (H) Hsp70 inhibits FLIP and IAP-2 expression. 293 cells were transiently transfected with His-Hsp70- or HA-IKKγ-expressing constructs. Forty-eight hours later, cells were treated with vehicle or TNF, and FLIP and IAP-2 expression were assayed by immunoblots 24 h later.
tective effects of these ZnCl₂-induced stress proteins are observed, however, in the IκBαDN and Hsp70-expressing cells, the combined lethal effects of increasing amounts of IκBαDN and Hsp70 plus TNF treatment lead to significant cell death and the small protective effects of ZnCl₂ are overwhelmed.

These results suggest that elevated Hsp70 sensitized cells to TNF killing by interfering with NF-κB signaling. If this were the case, activation of NF-κB would be expected to decrease apoptosis following combined exposure to Hsp70 and TNF. Hsp70 zinc-inducible cells were transiently transfected with NLS/p65 construct. Indeed, increasing levels of p65 improved cell survival following zinc induction of His-Hsp70 and TNF treatment [Fig. 6D]. To provide further evidence for this, we reversed the improved cell survival obtained with increasing levels of IKKγ by the IKKγ coiled-coil deletion mutant, IKKγCC [Fig. 6E].

To examine the mechanism by which Hsp70 inhibition of NF-κB signaling leads to cell death, we determined whether elevated Hsp70 favored TNF-mediated caspase activation via inhibiting the NF-κB pathway. The effects of Hsp70 and IκBαDN on caspase-3 processing were examined. The results showed that Hsp70 and IκBαDN, but not Hsp70C, decreased TNF-induced NF-κB activity [Fig. 6F, lower panel]. In addition, Hsp70 and IκBαDN significantly enhanced TNF-mediated PARP cleavage [Fig. 6F, topmost gel].

Enforced expression of p65/NF-κB or IKKγ did not entirely rescue Hsp70-mediated cell death [Fig. 6C,D]. This might be due to the low efficiency of transient transfection in Hela cells (~30%, data not shown). Additionally, Hsp70AS did not appear to block Hsp70 function efficiently [Fig. 1A,E]. We therefore performed additional studies in 293 cells, where much higher rates of transfection could be obtained (~90%–95% rate of transfection, data not shown). The 293 cells were transfected with IKKγ and Hsp70 siRNA or control GFPsiRNA for 2 d and then exposed to heat (30 min at 45°C) and/or TNF [50 ng for 15 min]. IKKγ activity was detected using an anti-P-IκBα antibody. Cell survival was measured using MTT 24 h after heat and/or TNF treatment. Treatment with GFPsiRNA alone, TNF plus GFPsiRNA, or heat shock plus GFPsiRNA did not affect cell survival [Fig. 6G, lanes 1–3]. The combination of TNF treatment and heat shock, however, decreased cell survival to ~30% [Fig. 6G, lanes 4,7]. Overexpression of HA-IKKγ (confirmed with HA-IKKγ antibody, Fig. 6G, third gel from top, lanes 5,6) enhanced IκBα phosphorylation [Fig. 6G, top gel, lanes 5,6] and increased cellular survival to near control levels of 90% [Fig. 6G, top panel, lanes 5,6]. Similarly, transfection of cells with Hsp70 siRNA almost reversed heat-shock-mediated IKK inhibition [Fig. 6G, top gel, lanes 7–9] and increased cell survival levels to near control levels of 90% despite combined heat shock and TNF treatment [Fig. 6G, top panel, lanes 7–9]. Lanes 4 and 7 in Figure 6G are replicates of each experiment and are not duplicates.

Because Hsp70 inhibits NF-κB transactivation and promotes caspase activation during TNF signaling [Fig. 6F], it is proposed that Hsp70 would block endogenous NF-κB-dependent antiapoptotic gene expression. Indeed, the results show that expression levels of cFLIP and IAP-2, two antiapoptotic proteins [Fig. 6H, upper two gels], decreased in TNF-treated 293 cells with increasing levels of His-Hsp70 protein. This Hsp70-mediated decrease of cFLIP and IAP-2 expression was IKKγ dependent because increasing IKKγ levels significantly reversed this [Fig. 6H, last three lanes].

Discussion

This study demonstrates how Hsp70 negatively regulates NF-κB, thus establishing the first direct, mechanistic link between Hsp70 and the NF-κB signaling cascade. The Hsp70 decrease of NF-κB activity tips the balance from cell survival to cell death following TNF/death receptor stimulation [Fig. 7]. The ability of Hsp70 to inhibit NF-κB signaling may contribute to the enhanced sensitivity of heated cells to chemotherapeutic agents and radiation treatment, and it helps explain at least some of the previous reports that demonstrate the proapoptotic effects of heat shock and Hsp70.

Hsp70 inhibits NF-κB activation by binding IKKγ

Previous studies have shown that Hsp70 and heat shock modulate NF-κB function, but the mechanism by which Hsp70 inhibits NF-κB remained unclear [Feinstein et al. 1997, Guzhova et al. 1997, Curry et al. 1999, Andres et al. 2001].

Because Hsp70 inhibits NF-κB transactivation and promotes caspase activation during TNF signaling [Fig. 6F], it is proposed that Hsp70 would block endogenous NF-κB-dependent antiapoptotic gene expression. Indeed, the results show that expression levels of cFLIP and IAP-2, two antiapoptotic proteins [Fig. 6H, upper two gels], decreased in TNF-treated 293 cells with increasing levels of His-Hsp70 protein. This Hsp70-mediated decrease of cFLIP and IAP-2 expression was IKKγ dependent because increasing IKKγ levels significantly reversed this [Fig. 6H, last three lanes].
al. 2002; Malhotra et al. 2002). In the present study, we clarify the mechanism by which Hsp70 inhibits NF-κB. Our data demonstrate that Hsp70 directly binds to IKKγ and this inhibits formation of the IKK complex and blocks NF-κB activation.

IKKγ contains several distinct domains that are involved in regulating IKK activity (Rothwarf et al. 1998; May et al. 2000; Yamamoto et al. 2001). The interaction of IKKγ with IKKα and IKKβ is critical for the assembly of the high-molecular-weight IKK complex that activates NF-κB, and IKKγ appears to function as an adaptor protein to increase the interactions of key factors required for NF-κB activation [Yamaoka et al. 1998]. IKKγ is an essential component of the IKK signalosome, as demonstrated in IKKγ-deficient or IKKγ mutant cells, which are unable to trigger the NF-κB response to a wide array of stimuli [Rothwarf et al. 1998; May et al. 2000; Rudolph et al. 2000; Makris et al. 2002]. The large 700–900-kDa IKK complex does not form in cells lacking IKKγ [Yamaoka et al. 1998]. A variety of proteins that interact with IKKγ, including RIP, A20, Tax, CIKS, vCLAP, and CYLD are involved in regulating NF-κB signaling [Jin et al. 1999; Leonardi et al. 2000; Poyet et al. 2000, 2001; Zhang et al. 2000; Brummelkamp et al. 2003]. We postulate and verify the possibility that Hsp70 binds IKKγ to hamper IKK activation. Our results show that Hsp70–IKKγ interaction plays a key role in NF-κB signaling.

It has been well characterized that the coiled-coil domain of IKKγ is responsible for IKKγ oligomerization, which is critical for activating IKK activity [Poyet et al. 2000]. The results of this study show that Hsp70 specifically binds the coiled-coil domain of IKKγ. These findings agree, at least in part, with those of Agou et al. [2002] who have shown that IKKγ [NEMO] binding via the coiled-coil domain to Hsp70 prevents incorrect interdomain pairing reactions. In the present study, we suggest that excess Hsp70 binding to IKKγ may prevent IKKγ self-association that is critical for the formation of the high-molecular-weight IKK complex. Hsp70 binding to the coiled-coil domain of IKKγ might lead to a chaperone-dependent change in the conformation of IKKγ. In this case, IKKγ does not form oligomers when bound to Hsp70, and it is rendered inaccessible to the IKKs and prevents assembly of the IKK complex. Because IKK activity is markedly impaired in cells that express IKKγCC or Hsp70, and enforced oligomerization of IKKγ was able to activate NF-κB [Poyet et al. 2000], these data indicate that IKKγ oligomerization is absolutely essential for TNF-induced NF-κB activation [Tegethoff et al. 2003].

Although strong evidence is provided that Hsp70 targeting to IKKγ plays a negative role in NF-κB signaling, the data do not rule out the possibility that Hsp70 may interact with other components of the IKK complex. The gel filtration data show that Hsp70 is detected in fractions in addition to the IKKγ fractions [200–500 kDa]. Future studies will be required to examine the possibility that Hsp70 could have other targets in this pathway. Interestingly, we found that IKKγ can be directly visualized in macromolecular foci in living cells, which is in line with previous reports [Poyet et al. 2000; Heussler et al. 2002]. Moreover, overexpression of Hsp70 or heat treatment significantly suppresses these macromolecular foci. These findings could suggest that Hsp70 inhibits these foci by preventing IKKγ oligomerization or Hsp70 suppresses these foci by inhibiting IKKγ binding to other proteins in the IKK complex.

Although Hsp70 did not bind directly to either IKKα or IKKβ [Fig. 3A], Hsp70 still influenced the formation of IKKα and IKKβ hetero- and homocomplexes [Fig. 4A]. It is likely that Hsp70 indirectly regulates the assembly of the IKK complex via interacting with IKKγ. Because IKKγ is a component of the IKKα and IKKβ hetero- and homocomplexes [Yamaoka et al. 1998; Mercurio et al. 1999], the formation of these complexes may be IKKγ dependent. Several reports demonstrate that IKKγ is required to facilitate the interactions of the IKK complex as a whole and/or that it influences individual components of the IKK complex [Mercurio et al. 1999; Poyet et al. 2000; Yamamoto et al. 2001]. The exact mechanism remains to be elucidated.

The finding that transfection of Hsp70 siRNA into 293 cells restores IKK activity is strong evidence, along with the other experiments shown, that Hsp70 directly regulates IKK activity. However, it is notable that complete elimination of Hsp70 expression with Hsp70 siRNA [Fig. 3E, lane 6] did not increase IKK activity over the non-heat-shock control, where moderate amounts of Hsp70 were present [Fig. 3E, lane 2]. This suggests that other heat shock proteins could also down-regulate IKK activity. Indeed, it has recently been shown that Hsp27 binds IKKβ and inhibits NF-κB activity [Park et al. 2003]. The situation is even more complex because Hsp90 can also bind the kinase domain of IKKα or IKKβ to form part of the ∼900-kDa IKK complex [Chen et al. 2002]. However, Hsp90 binding increased TNF-mediated NF-κB activation, as shown in the present study [Fig. 1A]. Therefore, although heat shock down-regulates NF-κB activity [Fig. 4D], this is likely due to a complex interaction of Hsp70–IKKγ and Hsp27–IKKβ to down-regulate IKK activity, whereas Hsp90–IKKα and Hsp90–IKKβ interactions up-regulate IKK activity. Because heat shock down-regulates NF-κB activation [Fig. 4D], the effects of Hsp70 and Hsp27 on down-regulation must overwhelm the up-regulation by Hsp90. In addition, the current studies demonstrate that although low levels of Hsp70 do not appear to affect IKK activity a great deal, high-level Hsp70 expression significantly blocks IKK activity and markedly decreases NK-κB activity.

Although the data show that Hsp70 specifically binds the coiled-coil motif of IKKγ, the regions of Hsp70 responsible for this binding are less clear. Although the ATP-binding domain of Hsp70 might be expected to bind IKKγ, our data show that neither the N-terminal nor the C-terminal domains of Hsp70 significantly affect NF-κB activity [Fig. 1A]. This result is similar to a recent study in which it was shown that full-length Hsp70 binds Apat-1, whereas neither Hsp70CC nor Hsp70N could be shown to interact with Apat-1 [Ravagnan et al. 2001]. The results of our study and that of Ravagnan et al. (2001) could suggest that full-length Hsp70 is essential.
for the interaction with some molecules such as IKK- 
and Apaf-1. This is not surprising because the ATP-bind-
ing domains and the peptide-binding domains of Hsp70 
are functionally coupled each other and probably essen-
tial for the complete repertoire of physiological effects 
of the molecule.

**Hsp70 promotes apoptosis by blocking NF-κB-dependent gene expression**

The discovery that Hsp70 suppressed NF-κB activation 
provides the first clear explanation for the pro-apoptotic 
effect of Hsp70 on cell survival. TNF applied to the three 
types of cells examined in this study activated NF-κB but 
did not produce apparent apoptosis, a finding consistent 
with the recent report [Micheau and Tschopp 2003]. TNF 
applied to the same three cell types overexpressing 
Hsp70 in this study, however, led to the failure to acti-
vate NF-κB, decreased expression of NF-κB antiapoptotic 
genes such as c-FLIP and IAP-2, and activated caspase-3-
deleted cleavage of PARP. These findings are in stark 
contrast with the effects of Hsp70 on mitochondrial-me-
diated apoptosis, in which Hsp70 inhibits cell apoptosis 
by interfering with Apaf-1 and activation of caspase-3-
mediated apoptotic pathways [Beere et al. 2000]. Hsp70 
appears to facilitate apoptosis that is initiated by TNF 
activation of its death receptors, with very high levels of 
Hsp70 protein being required to sensitize cells to TNF 
killing. These results are also generally consistent with a 
study that demonstrated increased cell death following 
imposition of antiapoptotic genes (Goyal et al. 2000).

The death-promoting effect reported here for Hsp70 is 
at variance with the commonly described protective ef-
effect of Hsp70. However, some reports show that heat 
shock can also increase susceptibility to death, as occurs 
for NK or LAK cells [Jaattela 1990; Fujieda et al. 1995]. 
In acute myeloid leukemia, apoptosis correlated with in-
creased Hsp70 levels [Chant et al. 1996]. A pro-apoptotic 
function of Hsp70 itself has been described after TCR/
CD3 or CD95 activation in Jurkat cells overexpressing 
Hsp70 [Liossis et al. 1997]. Hsp70 was found to acceler-
ate the caspase-activated DNase and DNA fragmenta-
tion in TCR-stimulated T-cell apoptosis [Liu et al. 2003]. 
It has been known for some time that heat produces 
radiosensitization, in which prior heat shock increases 
cell death in tumors produced by radiation [Dewey and 
Freeman 1980; Dewey 1994]. The adenovirus E1A sensi-
tizes tumor cells to lysis by macrophages through nitric 
oxide- and TNF-α-dependent mechanisms, despite up-
regulation of Hsp70 [Miura et al. 2003]. These data, to-
gether with our results, demonstrate that Hsp70 poten-
tiates TNF-mediated cell apoptosis. Although Hsp70 
generally prevents cell death, Hsp70 can promote cell 
death when it is overexpressed in a cell that is also ex-
posed to TNF and possibly other death-receptor mol-
ecules.

There is a report that TNF mediates susceptibility to 
heat-induced apoptosis by TNF-induced inhibition of 
Hsp70 expression [Schett et al. 2003]. However, this is 
inconsistent with the finding that TNF receptor I is re-
quired for induction of macrophage heat shock protein 
70 [Heimbach et al. 2001]. In addition, TNF induces 
Hsp70 expression in cardiac myocytes [Nakano et al. 
1996]. Therefore, although TNF could down-regulate 
Hsp70 in selected cells in certain circumstances [Schett 
et al. 2003], our data and other studies do not find that 
TNF inhibits HSFl/Hsp70 induction in most cells 
[Schett et al. 1998; Heimbach et al. 2001]. Instead, our 
data show that TNF-induced cell death is mediated at 
least potentiated by Hsp70 down-regulation of NF-κB 
signaling in most cells. The differences in these results 
might relate to differences in NF-κB-mediated gene ex-
pression in different cell types, where NF-κB mediates 
cell survival in neuronal and most other cells, whereas 
NF-κB may mediate cell death in at least some cell types.

Although most studies, including the present one, have 
shown that TNF and heat shock work synergisti-
cally to kill cells, the potential for sensitizing cells to 
TNF killing by manipulating Hsp70 may not be applic-
able to all cells, as evidenced by at least one report 
[Jaattela et al. 1998]. The discrepancy between these 
studies could be due to several experimental variables 
that have not been controlled for. For instance, recent 
data show that Hsp70 only temporarily protects against 
TNF-mediated cell apoptosis and this protection is lost 
after 16 h [Gabai et al. 2002]. Although one study has 
shown that Hsp70 inhibited TNF-induced ME180 cell 
death [Jaattela et al. 1998], another has found that Hsp70 
did not impede TNF-mediated ME180 cell apoptosis 
[Ravagnan et al. 2001]. It is certainly possible that el-
evated Hsp70 might protect cells from TNF-induced cell 
death, particularly during early stages. However, very 
high levels of Hsp70 do not favor cell survival when cells 
are exposed to TNF challenge, at least in this study [Fig. 
6A,C,G]. In addition, the current study shows that the 
Hsp70 effect on IKK-α is dose related, with lower Hsp70 
levels seemingly having little effect on IKK activity or 
NF-κB activation [Figs. 3E, 4D]. Only very high levels of 
Hsp70 protein decrease IKK activity [Fig. 4B], and the 
highest Hsp70 protein levels are required for the greatest 
TNF-induced apoptosis [Fig. 6B,C,G]. It is worth empha-
sizing that even in the current study, Hsp70 enhances 
TNF-induced apoptosis, but this did not occur for other 
stimuli [Fig. 6B]. Instead, Hsp70 provided modest protec-
tion against LPS-, PMA-, STS-, and serum deprivation-
induced cell death [Fig. 6B]. Taken together, these find-
ings indicate that the overexpression of Hsp70 is capable 
of either promoting or inhibiting apoptosis, depending 
on the nature of the stimulus. Finally, even our data 
suggest that zinc induction of other heat shock and other 
stress proteins may protect, at least to some degree, 
against TNF-induced cell death [Fig. 6C].

Our unique findings help explain a number of impor-
tant and puzzling phenomena that heat shock or el-
evated Hsp70 potentiate TNF-, FasL- and radiation-me-
diated cell death. It is likely that this Hsp70–NF-κB in-
teraction is involved in a variety of disease conditions. 
Clinical studies over a number of years have shown that 
there is often an advantage for using heat combined with 
radiation or cytotoxic drugs to enhance tumor cell kill-
Ran et al.

ing [Connor et al. 1977; Miller et al. 1977; Dewey and Freeman 1980; Dewey 1984; Curry et al. 1999]. We have identified the structural requirement for the interaction of Hsp70 with IKKγ and shown that the coiled-coil domain of IKKγ is necessary for TNF-triggered, Hsp70-dependent decrease of NF-κB activation. The coiled-coil domain of IKKγ thus appears to be an attractive target for drug development. Drugs like Hsp70 that target the coiled-coil domain of IKKγ might decrease NF-κB activation during death-receptor stimulation and be clinically useful for enhancing tumor cell death or controlling inflammation.

Materials and methods

Cell culture

Hela, Cos-1 [ATCC], and 293 [Invitrogen] cells were grown in DMEM medium [Gibco] supplemented with 10% [v/v] fetal calf serum and 2 mM L-glutamine [Gibco]. Hela Zn2+-inducible cell lines were generated by transfection with pMEP4, pMEP4/His-Hsp70, pMEP4/His-Hsp70C, and pMEP4/IκBαDN constructs. The transfected cells were selected with hygromycin [Gibco] for 2 wk. The selected clones were incubated in medium with Zn2+ for 4 h to induce gene expression that was confirmed by Western blot 24 h later.

Cell viability assays

The MTT assay was used to assess cell viability and was performed according to the directions in the manufacturer’s instruction manual [Sigma].

Plasmids and reagents

The following plasmids were used in various experiments in this study: pCMV/p65, pCMV4/IκBα (IκBα dominant negative), pCR-Flag/IKKα, pCR-Flag/IKKβ, pRC-HA/IKKα, pRC-Flag/IKKβ, pRC-HA/IKKγ, GST/IκBα (1–56), and pcDNA3/HSF1 (see Acknowledgments). Other plasmids were obtained commercially: pBluescript/Hsp90, pAG153/Hsp70 (ATCC), and pSV/B-galactosidase [Promega]; Cal4-Luc, NF-κB-Luc (Stratagene); and pMEP4/Invitrogen. The mammalian two-hybrid vectors pVP16 and pM, respectively. Hsp70C, and other constructs were in-frame inserted into the mammalian two-hybrid system and pEGFP and pDnRed2 were obtained from Clontech and pCrUHa was obtained from Santa Cruz.

The other plasmids were constructed by PCR or appropriate restriction-enzyme-digested methods: pcDNA3/Hsp90, pcDNA3/Hsp70 antisense, pcDNA3/IκBαDN, pcDNA3/His-Hsp70N [amino acids 1–420], pcDNA3/His-Hsp70C (amino acids 420–640), pMEP4/His-Hsp70, pMEP4/His-Hsp70C [amino acids 420–640], pM/IKKα, pVP16/IKKα, pM/IKKβ, pVP16/IKKβ, pM/IKKγ, pVP16/IKKγ, pM/IKKγCC (deleted amino acids 260–320, see Fig. 3B), pVP16/IKKγCC, pM/IκBα, pVP16/IκBα, pCrUHa/IKKγ, pCrUHa/IKKγN (amino acids 1–100), pCrUHa/IKKγC1 (amino acids 250–419), pCrUHa/IKKγC2 (amino acids 320–419), pCrUHa/IKKγCC, pM/p65AD, NLS/p65, GFP/IKKγ, GFP/IKKγCC, GFP/RFP/Hsp70, pRSET/Hsp70, pRSET/Hsp70C (amino acids 420–640).

Antibodies used included anti-His, phospho-IκBα [Cell Signaling], β-actin and TRITC-conjugated IgG, Flag [Sigma], HA, IκBα, IκKα/β (which recognize both IKKα and IKKβ), IκKγ, p65, c-FLIP, IAP-2 [Santa Cruz], and PARP [Oncogene]. Chemicals used included STS, TNF, LPS, PMA, protein G agarose beads, and Hoechst [Sigma]. The TNT kit that was used for in vitro translation and the Luciferase kits were from Promega. The SuperFect transfection kit was from Qiagen. The gel filtration column kit was from Amersham Pharmacia Biotech. The [γ-32P]ATP (3000 mCi/mmol) and [35S]methionine were from DuPont/NEN and the cross-linker EGS was from Pierce Inc.

Transfection and gene silencing assays

Hsp70 siRNA, IKKγ siRNA, and GFP siRNAs were generated as follows. The recombinant dice enzyme was used to cleave in vitro transcribed dsRNA into 22-bp siRNA according to the instructions supplied by the Dicer siRNA Generation Kit [Gene Therapy Systems, Inc].

The following primers were used for Hsp70: 5′-primer, 5′-GGCGTAATACGACTACTATAGGGAGATAATGATCGCGCGCTTCTCGTTG-3′; and 3′-primer, 5′-GGCGTAATACGACTACTATAGGGAGATAATGATCGCGCGCTTCTCGTTG-3′.

The following primers were used for IKKγ: 5′-primer, 5′-GGCGTAATACGACTACTATAGGGAGATAATGATCGCCTTCGAGAGACAGAGAGAGAGATGATCGCGCGCTTCTCGTTG-3′; and 3′-primer, 5′-GGCGTAATACGACTACTATAGGGAGATAATGATCGCGCGCTTCTCGTTG-3′.

Mammalian two-hybrid assays

IKKα, IKKβ, IKKγ, IKKγ mutants, IκBα, Hsp70, Hsp70N, Hsp70C, and other constructs were in-frame inserted into the mammalian two-hybrid vectors pVP16 and pM, respectively. Cos-1 cells were cotransfected with these constructs and the Gal4-Luc reporter gene and equal amounts of β-gal plasmid as an internal control. Luciferase activity was measured 24 h after transfections.

Reporter gene assays

Hela cell stable cell lines, 293 cells, and Cos-1 cells were seeded into 24-well plates. On the following day, cells were transfected using the SurpFect kit. Each well was transfected with 50 ng pSV/B-galactosidase [Promega]; Cal4-Luc, NF-κB-Luc (Stratagene); and pMEP4/Invitrogen. The mammalian two-hybrid system and pEGFP and pDnRed2 were obtained from Clontech and pCrUHa was obtained from Santa Cruz.

The 32P-labeled oligonucleotide AGTTGGGGACTTTCCCA was used as the probe. Probe (2.5 ng), nuclear proteins (5 µg), and 32P-labeled [γ-32P]ATP (3000 mCi/mmol) were incubated in binding buffer at 4°C for 1 h. The mixtures were washed five times with wash buffer to remove nonspecifically bound proteins and then analyzed by SDS-PAGE and autoradiography.

In vitro protein interactions

Three microliters of 35S-labeled-IKKα, IKKβ, IKKγ, and IKKγ mutants and 0.25 µM of Hsp70 or Hsp70C previously immobilized on beads were incubated in binding buffer for 3 h at 4°C. The mixtures were washed five times with wash buffer to remove nonspecifically bound proteins and then analyzed by SDS-PAGE and autoradiography.
**Gel filtration chromatography**

Gel filtration chromatography was carried out on a Superose 6 column. Hsp70 or Hsp70C stably expressing cells were transiently transfected with Flag-tagged IKKα, Flag-tagged IKKβ, and HA-tagged IKKγ. Cells (2 x 10^7) were washed with PBS. Cells were collected, and 500 μL extracts were prepared as previously described (Polet et al. 2000). The cell extracts were loaded onto a Superose 6 column. The column was precalibrated with the molecular mass marker proteins dextran (2000 kDa), thyroglobulin (670 kDa), β-amylase (230 kDa), and bovine serum albumin (66 kDa). Proteins were eluted from the column and analyzed by SDS-PAGE followed by Western blotting for the different IKK subunits and Hsp70 and Hsp70C using appropriate antibodies. IKK activity of each fraction was measured using an in vitro kinase assay.

**Immunoprecipitation, immunoblotting, and kinase assays**

Immunoprecipitation studies were performed as described by Chen et al. (2002). Briefly, cells were collected and lysed in 500 μL lysis buffer. Cleared cell extracts were incubated in 2 μg/500 μL of the appropriate antibodies and 10 μL of protein-G agarose beads for 3 h at 4°C. After the incubation, the precipitated complexes were washed five times and samples were boiled for 5 min in loading buffer, applied to SDS-PAGE, and analyzed by Western blotting using appropriate antibodies. IKKα and IKKβ were immunoprecipitated from cell extracts with the appropriate antibodies, as indicated in the figure legends. In vitro kinase assay of the immunocomplexes was performed as described by Chen et al. (2002).

**Chemical cross-linking of proteins**

293 cells were cultured in DMEM without methionine for 1 h and then labeled with 500 μCi of [35S]-methionine per 60 mm dish for 6 h. The cells were lysed in lysis buffer. Cross-linking using EGS and immunoprecipitation were performed as recently described (Tegelhoff et al. 2003). Cross-linking of in vitro translated IKKγ was performed using the same methods.

**Confocal microscopy**

Microscopy was performed using a Zeiss LSM-510 confocal microscope.

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