Hsp90 Interaction with INrf2(Keap1) Mediates Stress-induced Nrf2 Activation*

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INrf2(Keap1) functions as an adapter for Cul3/Rbx1-mediated degradation of Nrf2. In response to stress, Nrf2 is released from INrf2 and translocates inside the nucleus leading to activation of cytoprotective proteins critical in protection against adverse effects including cancer. We demonstrate here a novel role of heat shock protein 90 (Hsp90) in control of the INrf2 and Nrf2 activation. Hsp90 interacts with INrf2 that leads to stabilization of INrf2 during heat shock stress. Domain mapping showed the requirement of INrf2-NTR and the Hsp90-CLD region for interaction of Hsp90 with INrf2. Heat shock and antioxidant stress showed the requirement of INrf2-NTR and the Hsp90-CLD region for interaction of Hsp90 with INrf2. Heat shock or antioxidant stress subsidized, thereby allowing downstream signaling. INrf2 is released from Hsp90 once the heat shock or antioxidant stress induced dissociation of INrf2-Thr55. This led to increased Hsp90-INrf2 interaction, dissociation of the Rbx1/Cul3-INrf2/Nrf2 complex, and activation of Nrf2. Inhibitors of CK2 and Hsp90, and mutants of INrf2-Thr55 abolished the Hsp90-INrf2 interaction and downstream signaling. INrf2 is released from Hsp90 once the heat shock or antioxidant stress subsidized dissociation of INrf2 to interact with Nrf2 and facilitate ubiquitination and degradation. The results together demonstrate a novel role for the stress-induced Hsp90-INrf2 interaction in regulation of Nrf2 activation and induction of cytoprotective proteins.

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□The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S5.

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The mechanisms by which Nrf2 are released from INrf2 under stress have been actively investigated. One mechanism is that cysteine thiol groups of INrf2 act as sensors of oxidative stress and are modified by the chemical inducer causing formation of disulfide bonds between cysteines of two INrf2 peptides. This results in a conformational change that renders INrf2 unable to bind to Nrf2 (6, 7). On the other hand, we and others have shown that antioxidant-induced protein kinase Cδ (PKCδ) phosphorylates Nrf2Ser40 leading to dissociation of Nrf2 from INrf2, which stabilizes Nrf2 and allows it to translocate in the nucleus (2, 8, 9). More recently, we demonstrated that the two mechanisms work in concert with each other (2). The Nrf2 in the nucleus binds with the antioxidant response element (ARE) of the promoter region of antioxidant genes and increases their expression. Nrf2 up-regulates several genes encoding phase II detoxification enzymes and antioxidant proteins, such as NAD(P)H:quinoine oxidoreductase-1 (NQO1), γ-glutamylcysteine synthetase, heme oxygenase 1, thioredoxin reductase-1, and the transcriptional activation of Nrf2 and its downstream genes have been shown to be critically important for resistance to oxidative stress- and chemotherapy, as well as many drug-resistant and normal tissues (1, 11, 12).

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Heat shock protein 90 (Hsp90) is a molecular chaperone and is one of the most abundant proteins expressed in cells (20). Hsp90 is a member of the heat shock protein family up-regulated in response to stress. In unstressed cells, Hsp90 plays a number of important roles, which include assisting in folding, intracellular transport, maintenance, and degradation of proteins, as well as facilitating cell signaling (21). Hsp90 is known to associate with the non-native structures of many proteins that have led to the proposal that Hsp90 is involved in protein folding in general. Furthermore, Hsp90 has been shown to suppress the aggregation of a wide range of “client” or “substrate” proteins and hence acts as a general protective chaperone (22, 23). However, Hsp90 is somewhat more selective than other chaperones. For example, many cancer cells overexpress a number of proteins involved in cell survival including PI3K and AKT. Inhibition of these two proteins triggers apoptosis. Hsp90 stabilizes the PI3K and AKT proteins. Hence inhibition of Hsp90 appears to induce apoptosis through inhibition of the PI3K/AKT signaling pathway (24). Another important role of Hsp90 in cancer is stabilization of mutant proteins such as v-Src, the fusion oncogene Bcr/Abl, and p53 that appear during cell transformation. It appears that Hsp90 can act as a “protector” of less stable proteins produced by DNA mutations (25).

In the present study, we demonstrated a novel role of Hsp90 in regulation of INrf2-Nrf2 signaling and induction of chemopreventive proteins. We show that heat shock and antioxidant stress induce Hsp90 and INrf2. In addition, CK2 phosphorylates INrf2 Thr55, which induces Hsp90. This interaction requires INrf2 and Hsp90 and degrades INrf2. Hsp90-INrf2 interaction during heat shock or by exposure to cells leads to dissociation of the Rbx1/Cul3-INrf2 complex and release or activation of Nrf2 and Nrf2 downstream gene expression. INrf2 dissociates from Hsp90 when heat shock or antioxidant stress subsides and interacts with Nrf2 for Nrf2 degradation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—INrf2 and mutants were previously described (26). The INrf2T55A mutant was generated using the Gene Tailor site-directed mutagenesis kit (Invitrogen). The various domain deletion mutants of the Hsp90 protein were cloned into pcDNA3.1/V5-His/Topo vector by TA cloning. The primer sequences used for construction of the various Hsp90 mutant plasmids and FLAG-INrf2T55A are shown in supplemental Table S1. The construction of pcmx-FLAG-INrf2, pcDNA-INrf2-V5, and PGL2b-NQO1-ARE have been described previously (26). All plasmids were confirmed by DNA sequencing.

**Cell Culture**—Mouse hepatoma (Hepa-1) cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (40 units/ml), and streptomycin (40 µg/ml) in an incubator at 37 °C in 95% air and 5% CO₂. Hepa-1 cells were treated with DMSO or tBHQ (50 µM). CK2 inhibitor II, obtained from Sigma...
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The cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, and 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) supplemented with protease inhibitor mixture (Roche Applied Science). Cytoplasmic and nuclear fractions were prepared using the Active Motif nuclear extract kit (Active Motif, Carlsbad, CA). 60–80 μg of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. For immunoblotting, antibodies used were anti-INrf2 antibody (Abcam), anti-CK2 antibodies (Cell Signaling Boston, MA), anti-Rbx1 antibody (BIOSOURCE), and γ-glutamylcysteine synthetase (GCLC) antibody (Abcam) were also used. Anti-FLAG-HRP (1:10,000), anti-HA-HRP (1:10,000), and anti-β-actin (1:10,000) antibodies were obtained from Sigma and anti-GFP and anti-V5-HRP from Invitrogen. The membranes were washed and immunoreactive bands were visualized using a ECL chemiluminescence system (Amersham Biosciences). To confirm the purity of the nuclear-cytoplasmic fractionation, the membranes were re-probed with cytoplasm-specific anti-lactate dehydrogenase and nuclear specific anti-lamin B antibodies.

Immunoprecipitation (IP) and Immunoblot Analysis—Hepa-1 cells were transfected with the indicated plasmids for 36 h or control 293 cells or FLAG-INrf2–293 T-REx HEK293 cells expressing tetracycline-inducible INrf2 were used. The cells were harvested after growing in 10% FBS containing 1 mM sodium vanadate, a serine/threonine phosphatase inhibitor, and protease inhibitors (Roche Applied Science). After immunoprecipitation, 1 mg of the same cell lysates were pre-cleared by protein AG Plus-agarose (Santa Cruz Biotechnology) and extracts were incubated with anti-INrf2 antibody or anti-phosphothreonine antibody (Stressgen), and visualized using a ECL chemiluminescence system (Amersham Biosciences).

Immunofluorescence—Hepa-1 cells were grown in Lab-Tek II chamber slides and separately transfected with INrf2-GFP and INrf2ΔNTR-2XGFP or INrf2ΔNTR-GFP for 32 h, fixed in 2% formaldehyde, and permeabilized by treatment with 0.25% Triton X-100. Cells were washed twice with PBS and incubated with 1:1000 dilution of mouse Hsp90 primary antibodies in 2% BSA for 12 h at 4 °C. The cells were then washed twice with PBS and incubated with Alexa Fluor 594-conjugated anti-mouse secondary antibodies (Invitrogen). Similarly, Hepa-1 cells were also transfected with Hsp90-V5 and deletion plasmids tagged with V5, fixed, permeabilized, and incubated with anti-V5-FITC antibody and goat anti-V5-FITC antibody (Stressgen), and visualized using a ECL chemiluminescence (Amersham Biosciences).

Transient Transfection and Luciferase Assay—Hepa-1 cells were transfected with 1 μg of the indicated plasmids using Effectene transfection reagent (Qiagen). 36 h after transfection,
the cells were harvested and protein expression was examined by immunoblotting. For luciferase reporter assay, Hepa-1 cells were co-transfected with 0.1 \( \mu \)g of human NQO1 promoter ARE-Luc reporter plasmid and 10 times less quantities of firefly Renilla luciferase encoded by plasmid pRL-TK. After 24 h of transfection, the cells were either treated with DMSO or tBHQ at 37 °C or incubated for different times at 42 °C for heat shock as indicated in figures. Cells were lysed and analyzed for luciferase activity by previously described procedures (27).

**siRNA Interference Assay**—Hsp90 siRNA was used to inhibit Hsp90 protein by a procedure described previously (27). Hsp90 siRNA, and control siRNA were purchased from Dharmacon. In related experiments, Hepa-1 cells were transfected with 25 to 100 nM Hsp90 siRNA or control siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Thirty-two h after transfection, cells were harvested and Hsp90, INrf2, Nrf2, and actin protein levels were analyzed by immunoblotting.

**Mass Spectrometry Analysis of INrf2-Hsp90 Interaction and Identification Phosphorylation Sites of INrf2**—Control 293 cells or FLAG-INrf2–293 cells were treated with tetracycline (0.5 \( \mu \)g/ml) for 24 h to induce FLAG-INrf2 protein in FLAG-INrf2–293 cells. 10 mg of cell lysates were immunoprecipitated with anti-FLAG antibodies, the immune complexes were separated by SDS-PAGE, and gels were stained with Coomassie Brilliant Blue. Gel slices containing bands indicated by arrows in figure were reduced, alkylated, and digested with trypsin. Tryptic peptides were desalted and subjected to LC-MS/MS analysis by the UMB Proteomics Core using a Voyager DEPro with a 20-Hz 337-nm nitrogen laser (Applied Biosystems, Foster City, CA). The Mascot software package was used to match the mass of the peptides with the predicted tryptic peptides generated from the translated human genome and Hsp90 as an interacting protein of INrf2 was identified from INrf2–293 cells. For INrf2 phosphorylation studies, INrf2–293 cells were treated with tetracycline for 24 h followed by treatment with DMSO or tBHQ (4 h). Ten mg of whole cell lysates were immunoprecipitated with anti-FLAG antibodies, the immune complexes were separated by SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. INrf2 bands were excised, cut into \( \frac{1}{10} \) mm pieces, digested with trypsin, subjected to reverse phase liquid chromatography, and analyzed. All MS analyses were performed using an LCQ Deca (Thermo Scientific, Waltham, MA) mass spectrometer equipped with a nano-spray ionization source. Peptides were introduced into the mass spectrometer via a 75-\( \mu \)m inner diameter/15-\( \mu \)m tip
**RESULTS**

**Hsp90 Interacts with INrf2**—We successfully generated stable FLAG-INrf2-HEK293 cells that expressed FLAG-INrf2 protein upon exposure of cells to tetracycline (26). The cell lysates from untreated and tetracycline-treated control 293 and FLAG-INrf2-293 cells were immunoprecipitated with anti-FLAG antibody to identify INrf2 interacting proteins. The results showed that INrf2 interacted with Hsp90 protein. Furthermore, the interaction of FLAG-INrf2 and Hsp90-His-tagged protein was analyzed by pull-down experiments. Hepa-1 cells lysates co-transfected with pcDNA3.1Hsp90-His and FLAG-INrf2 plasmids were mixed with protein AG-agarose beads or Ni-NTA beads and His-tagged Hsp90 protein was pulled down. The beads were boiled with SDS dye and immunoblotted with anti-Hsp90 antibody (Fig. 1C). The data demonstrated that Ni-NTA beads and not protein AG-agarose beads were pulled down by Hsp90-His-tagged protein, and Hsp90 protein pulled down by FLAG-INrf2 protein (Fig. 1D). These results together confirmed that INrf2 interacts with Hsp90.

**INrf2 through Its NTR Domain Interacts with the CLD Domain of Hsp90**—Next we mapped the interacting domains of INrf2 and Hsp90 proteins. INrf2 possesses 5 discrete domains designated as NTR (N-terminal region; 1–60 amino acids),
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**FIGURE 6. Hsp90 stabilizes INrf2.** A, immunoblot analysis of the effect of overexpression of Hsp90 on INrf2 of Hepa-1 cells were transfected with pcDNA or increasing concentrations of Hsp90-V5 plasmid. The levels of Hsp90-V5 and endogenous INrf2 were analyzed by immunoblot analysis of the lysate probed with anti-INrf2 and anti-actin antibodies (left panel). IP and immunoblot analysis of the INrf2 and Hsp90 bands (Fig. 2, right panel) were probed with anti-INrf2 and anti-Hsp90 antibodies. B, immunoblot analysis of the lysates that were transfected with control siRNA or Hsp90-specific siRNA and Hsp90, INrf2, and actin levels were analyzed by immunoblotting with anti-Hsp90, anti-INrf2, and anti-actin antibodies (right panel). C, immunoblot analysis of the left panel, Hepa-1 cells were treated with DMSO or the Hsp90-specific inhibitor geldanamycin (2 μM) for an additional 4 h. FLAG-INrf2 protein was purified by anti-FLAG beads, the proteins were separated by SDS-PAGE and stained with Coomassie Blue. INrf2 bands were sliced and subjected to mass spectrometry for phosphorylation analysis.

**BTB (broad complex, tamtrac and bric-a-brac) and IVR (linker region; 599–624 amino acids)** were cloned in a 2× GFP vector. Similarly, individual domains of INrf2 were also cloned in 2× GFP vector. Hepa-1 cells co-transfected with FLAG-tagged INrf2 and Hsp90 mutants, Hsp90-V5 plasmid, and transfected with anti-GFP and Hsp90 antibodies (Fig. 3, panels B and C). Immunoblot analysis demonstrated expression of the correct size INrf2 of the mutant and Hsp90 bands (Fig. 3, panels C, left panels). The forward and reverse immunoprecipitation results demonstrated that full-length INrf2 and all domain deletion mutants except INrf2ΔNTR interacted with the Hsp90 protein (Fig. 3B, right panels). The immunoprecipitation results also showed that only full-length INrf2 and the NTR domain of INrf2 interacted with Hsp90 (Fig. 3C, right panels). These results suggested that the NTR domain is required for the INrf2-Hsp90 interaction. Immunocytochemistry analysis supported the immunoprecipitation results (supplemental Fig. S1). Full-length INrf2 and not INrf2ΔNTR showed interaction. These results together with the immunoprecipitation results confirmed that the INrf2-NTR domain interacts with Hsp90.

Hsp90 protein consists of 4 unique domains that include the N-terminal domain (NTD), charged linker domain (CLD), middle domain (MD), and the C-terminal domain (CTD) (Fig. 3A). We successfully generated N and C terminus domain deletion mutants of Hsp90 to investigate which domain of Hsp90 is required for interaction with INrf2 (Fig. 3A). The various Hsp90 domain deletion mutants were co-transfected with FLAG-INrf2 in Hepa-1 cells. The various plasmids bearing Hsp90 mutants produced the expected size of proteins (Fig. 3B, left panel). Forward and reverse immunoprecipitations and immunoblot analysis was performed to analyze the interaction of full-length and deletion mutants of Hsp90 (Fig. 3B, right panel). The results demonstrate that the Hsp90 protein deficient in NTD and CLD failed to interact with INrf2. Furthermore, immunoprecipitation results also with endogenous INrf2 (Fig. 3B, right panel, top panel). The above results together concluded that the full-length INrf2 and not INrf2ΔNTR showed interaction. The above results together concluded that the NTR domain is required for the INrf2-Hsp90 interaction. Immunocytochemistry analysis supported the immunoprecipitation results (supplemental Fig. S1). Full-length Hsp90-V5, and not NTD-MD-CLD-Hsp90 or NTD∆CLD-Hsp90 or NTD-CLD-Hsp90 (Fig. 3B, right panel, lower two blots). Furthermore, immunoblot analysis supported the immunoprecipitation results (supplemental Fig. S2). Full-length Hsp90-V5, and not NTD-MD-CLD-Hsp90 or NTD∆CLD-Hsp90 or NTD-CLD-Hsp90 (Fig. 3B, right panel, lower two blots). Furthermore, immunoblot analysis supported the immunoprecipitation results (supplemental Fig. S2). Full-length Hsp90-V5, and not NTD-MD-CLD-Hsp90 or NTD∆CLD-Hsp90 or NTD-CLD-Hsp90 (Fig. 3B, right panel, lower two blots). Furthermore, immuno-
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Heat shock and antioxidant-induced Hsp90, which stabilizes INrf2. A, immunoblot analysis showed the effect of heat shock on stabilization of INrf2. Hepa-1 cells were incubated at 37 or 42 °C for the indicated time periods, lysed, and 60 μg of cell lysates were immunoblotted with anti-Hsp90, anti-INrf2, and anti-actin antibodies. B, IP and immunoblot analysis of INrf2 threonine phosphorylation and Hsp90-INrf2 interaction during heat shock. Left panels indicate threonine phosphorylation of INrf2 and Hsp90-V5 protein. C, IP and immunoblot analysis revealed that a parallel increase in Hsp90 and INrf2 resulted in enhanced interaction between Hsp90-V5 and INrf2 (Fig. 6B). The increase in INrf2 was parallel to the increase in Hsp90 and suggested that Hsp90 stabilized INrf2. Forward and reverse IP and immunoblot analysis revealed that a parallel increase in Hsp90 and INrf2 resulted in enhanced interaction between Hsp90-V5 and INrf2 (Fig. 6A, right panel). Similarly, siRNA inhibition of Hsp90 led to a concentration-dependent decrease in INrf2 (Fig. 6B). In related experiments, treatment of Hepa-1 cells with the Hsp90 inhibitor geldanamycin also led to a significant decrease in INrf2 (Fig. 6C, left panel). IP and immunoblot analysis demonstrated that a decrease in Hsp90 activity in geldanamycin-treated cells also led to a significant loss of INrf2-Hsp90 interaction (Fig. 6C, right panel). These results together suggested that alterations and inactivation in Hsp90 cause similar alterations in INrf2-Hsp90 interaction and INrf2 stability. The results also reveal that Hsp90 interaction with INrf2 leads to stabilization of INrf2.

Heat Shock and Antioxidant Induce Hsp90/CK2, INrf2Thr Phosphorylation, and Hsp90-INrf2 Interaction—We analyzed the role of Hsp90-INrf2 interaction in heat shock and antioxidant stress. Hepa-1 cells were incubated at 37 and 42 °C for different time intervals and analyzed for Hsp90, INrf2, and Hsp90-INrf2 interaction (Fig. 7, A–C). Immunoblot analysis revealed that heat shock at 42 °C led to a time-dependent increase in Hsp90, INrf2, and CK2 as compared with cells incubated at 37 °C. A, IP and immunoblot analysis of INrf2 threonine phosphorylation and Hsp90-INrf2 interaction during heat shock. Left panels indicate threonine phosphorylation of INrf2 and Hsp90-V5 protein. C, IP and immunoblot analysis revealed that a parallel increase in Hsp90 and INrf2 resulted in enhanced interaction between Hsp90-V5 and INrf2 (Fig. 6B). The increase in INrf2 was parallel to the increase in Hsp90 and suggested that Hsp90 stabilized INrf2. Forward and reverse IP and immunoblot analysis revealed that a parallel increase in Hsp90 and INrf2 resulted in enhanced interaction between Hsp90-V5 and INrf2 (Fig. 6A, right panel). Similarly, siRNA inhibition of Hsp90 led to a concentration-dependent decrease in INrf2 (Fig. 6B). In related experiments, treatment of Hepa-1 cells with the Hsp90 inhibitor geldanamycin also led to a significant decrease in INrf2 (Fig. 6C, left panel). IP and immunoblot analysis demonstrated that a decrease in Hsp90 activity in geldanamycin-treated cells also led to a significant loss of INrf2-Hsp90 interaction (Fig. 6C, right panel). These results together suggested that alterations and inactivation in Hsp90 cause similar alterations in INrf2-Hsp90 interaction and INrf2 stability. The results also reveal that Hsp90 interaction with INrf2 leads to stabilization of INrf2.
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**FIGURE 8.** Heat shock and antioxidant-induced dissociation of Rbx1/Cul3-INrf2-Nrf2 complex, sequestering of INrf2, and Nrf2 release and activation. A, left panel. IP and immunoblot analysis of the effect of heat shock on Rbx1/Cul3-INrf2-Nrf2 complex. Hepa-1 cells were incubated at 37 or 42 °C for 1, 3, and 7 h. Cells were lysed and 1 mg of cell lysates were immunoprecipitated with anti-INrf2 antibody (left input panel). IP and immunoblot analysis of the effect of heat shock on the Hsp90-INrf2 interaction. Hsp90-INrf2 complexes were immunoblotted with anti-Hsp90, anti-Cul3, anti-Rbx1, anti-Nrf2, and anti-INrf2 antibodies as indicated. B, middle panel. IP and immunoblot analysis of the effect of tBHQ on Rbx1/Cul3-INrf2-Nrf2 complex. Hepa-1 cells were treated with DMSO for 8 h or tBHQ for the indicated time periods, and 60 μg of cell lysates were immunoprecipitated with anti-INrf2 antibody (left input panel). IP and immunoblot analysis of the effect of tBHQ on the Hsp90-INrf2 interaction. Hsp90-INrf2 complexes were immunoblotted for Hsp90, Cul3, Rbx1, and Nrf2 in Hepa-1 cells exposed to 42 °C compared with 37 °C incubated cells (Fig. 8B, middle panel). Reverse IP with anti-Hsp90 antibodies demonstrated increased interaction of Hsp90 with INrf2 and no interaction of Hsp90 with Rbx1/Cul3 and Nrf2 in cells exposed to heat shock at 42 °C (Fig. 8B, right panel). Anti-oxidant tBHQ demonstrated similar results as observed with heat shock (Fig. 8B). Antioxidant treatment led to increased interaction of Hsp90 with INrf2, loss of interaction of INrf2 with Cul3/Rbx1, and no interaction between INrf2 and Nrf2 (Fig. 8B, middle and right panels). The immunoblotting data also suggested that during heat shock or antioxidant treatments, Nrf2 was released from INrf2, and led to induction of NQO1 expression (Fig. 8, A and B, left panels). Indeed, all results combined suggest that antioxidant (tBHQ) treatments to cells increased nuclear...
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Nrf2 downstream GCLC and NQO1 gene expression. These results are consistent with the results from earlier in our study. Interestingly, removal of heat shock by incubation at 37 °C for 8 h led to restoration of Hsp90, INrf2, and Nrf2 to normal levels and increased the GCLC and NQO1 proteins to basal and constitutive levels. However, Nrf2 downstream genes continued to express at higher levels at 8 h of transfer of cells from 42 to 37 °C (Fig. 10A). The same Hepa-1 cell lysates were also subjected to IP and immunoblot analysis to investigate INrf2Thr phosphorylation and interaction of INrf2 with Hsp90 and Nrf2 (Fig. 10A, right panel). The results showed that removal of heat shock (8 h) led to a decrease in INrf2Thr phosphorylation and increased interaction of INrf2 with Hsp90 interaction, and decreased INrf2 interaction with Nrf2 (Fig. 10A, right panel). The data demonstrate that removal of heat shock leads to an increase in Hsp90-INrf2 interaction and decreased INrf2 interactions that leads to restoration of basal levels of the various proteins.

![Diagram](link)

**Removal of Heat Shock or Antioxidant (tBHQ) Leads to Down-regulation of Hsp90 and Restoration of Normal Interaction with INrf2**—Hepa-1 cells were incubated either at 37 or 42 °C for 7 h. Two sets of cells exposed to heat shock (42 °C for 7 h) were returned for incubation at 37 °C for another 4 and 8 h and cell lysates were immunoblotted with Hsp90, INrf2, Nrf2, GCLC, and NQO1 antibodies (Fig. 10A, left panel). The results demonstrated that heat shock exposure led to an increase in Hsp90 and stabilization of INrf2, and an increase in Nrf2 and localization of Nrf2, respectively. Downstream genes continued to express at higher levels at 8 h of transfer of cells from 42 to 37 °C (Fig. 10A). The same Hepa-1 cell lysates were also subjected to IP and immunoblot analysis to investigate INrf2Thr phosphorylation and interaction of INrf2 with Hsp90 and Nrf2 (Fig. 10A, right panel). The results showed that removal of heat shock (8 h) led to a decrease in INrf2Thr phosphorylation and increased interaction of INrf2 with Hsp90 interaction, and decreased INrf2 interaction with Nrf2 (Fig. 10A, right panel). The data demonstrate that removal of heat shock leads to an increase in Hsp90-INrf2 interaction and decreased INrf2 interactions that leads to restoration of basal levels of the various proteins.

![Diagram](link)
role of the DGR region of INrf2 that is involved in binding to Nrf2 in Nrf2/ARE-mediated gene expression is well established (10). The crystal structure reveals that Hsp90 consists of three highly conserved domains, the N-terminal ATP-binding domain (25 kDa), a middle domain (35 kDa), and a C-terminal dimerization domain (12 kDa) (28–30). Hsp90 exists as a homodimer (31). The N-terminal domain contains a specific ATP binding pocket, which has been well characterized (28, 31). The middle domain is highly charged and its major role is to distinguish various types of client proteins and adjust the molecular chaperone for proper substrate activation (32). The C-terminal domain strengthens the weak association between the two N-terminal domains of the Hsp90 dimer. A second ATP-binding site is located in the C terminus, which does not exhibit ATPase activity (33). The molecular chaperone, Hsp90, facilitates the maturation and/or activation of over 100 client proteins involved in signal transduction and transcriptional regulation (34). Interestingly, results demonstrated that INrf2 interaction with Hsp90 required CK2 phosphorylation of INrf2Thr55. Mutation of Thr-55 to alanine significantly reduced threonine phosphorylation of INrf2 and interaction of INrf2 with Hsp90. In addition, chemical inhibition of CK2 also led to a decreased interaction of INrf2 with Hsp90.

Further studies on the INrf2-Hsp90 interaction led to the discovery of a novel mechanism of heat shock-mediated release of Nrf2 from INrf2 and activation of Nrf2 downstream gene expression. Interestingly, antioxidant was also able to recruit this mechanism for delayed release of Nrf2 and activation of Nrf2 downstream gene expression. Under normal conditions, a low level of INrf2-Hsp90 interaction is observed. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2. Heat shock and exposure to antioxidants (2) reversed INrf2-Hsp90 interaction and increased INrf2-Hsp90 interaction with CK2.
8 h and involves induction of Hsp90 and CK2, CK2 phosphorylation of INrf2Thr55, Hsp90 interaction with phospho-INrf2Thr55 to sequester INrf2, destabilization of Rbx1/Cul3-INrf2-Nrf2, uninhibited nuclear localization of Nrf2, and induction of downstream gene expression. In other words, the Hsp90/CK2 mechanism prolongs antioxidant activation of Nrf2. Interestingly, heat shock stress has only a delayed phase of Nrf2 activation presumably due to the absence of chemical modification reactions, a characteristic of the early phase of activation (supplemental Fig. S5).

The present studies demonstrated Hsp90 regulation of INrf2/Nrf2 signaling. This raised an interesting question regarding INrf2 regulation of Hsp90 in normal and stressed conditions because both interacted with each other. The Hsp90 chaperone protein is a remarkably versatile protein involved in stress response and in normal homeostatic control mechanisms (35, 36). It interacts with protein kinases, transcription factors, and other proteins, and either facilitates their stabilization as observed for INrf2 or directs them for proteasomal degradation. By this means, Hsp90 displays a multifaceted ability to influence signal transduction, chromatin remodeling, and epigenetic regulation, development and morphological evolution in influence signal transduction, chromatin remodeling, and epigenetic regulation, development and morphological evolution in cancer. INrf2 titration of Hsp90 might or might not influence these processes and remains to be investigated.

In conclusion, we demonstrated that heat shock and antioxidant treatment induced Hsp90 and CK2. The phospho-INrf2Thr55. This led to a required INrf2 interaction, dissociation of the complex, release of Nrf2, and antioxidant gene expression. Hsp90, INrf2, and Hsp90-INrf2 interaction, CK2, phosphor-INrf2Thr55, and INrf2 phosphorylation, Nrf2, and Nrf2 downstream gene expression all return antioxidant stress subsided. INrf2 interacts with Nrf2 and facilitates INrf2Thr phosphorylation, Nrf2, and Nrf2 downstream gene expression. Hsp90, INrf2, Hsp90-INrf2 interaction, CK2, and antioxidant stress subsided. INrf2 interacts with Nrf2 and facilitates INrf2Thr phosphorylation, Nrf2, and Nrf2 downstream gene expression.