Heat Shock Protein 90α Recruits FLIP<sub>S</sub> to the Death-Inducing Signaling Complex and Contributes to TRAIL Resistance in Human Glioma

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Abstract

Heat shock protein 90 (HSP90) is a molecular chaperone that contributes to the proper folding and stability of target proteins. Because HSP90 has been suggested to interact with FLIP<sub>S</sub>, the key regulator of tumor necrosis factor-α–related apoptosis–inducing ligand (TRAIL)–induced apoptosis in glioma cells, we examined the role HSP90 played in controlling TRAIL response. HSP90α was found to associate with FLIP<sub>S</sub> in resting cells in a manner dependent on the ATP-binding NH<sub>2</sub>-terminal domain of HSP90α. Following TRAIL exposure, HSP90α and the client FLIP<sub>S</sub> protein were recruited to the death-inducing signaling complex (DISC). Short interfering RNA–mediated suppression of HSP90α did not alter the total cellular levels of FLIP<sub>S</sub>, but rather inhibited the recruitment of FLIP<sub>S</sub> and other antiapoptotic proteins such as RIP and FLIP<sub>L</sub> to the DISC, and sensitized otherwise resistant glioma cells to TRAIL-induced apoptosis. These results show that HSP90α is required for FLIP<sub>S</sub> recruitment to the DISC and that HSP90α can modulate the antiapoptotic effects of FLIP<sub>S</sub>.

Introduction

Heat shock proteins (HSP) are a highly conserved group of intracellular proteins classified by molecular weight into groups of HSP110, HSP90, HSP70, HSP60, small molecular HSPs (<27 kDa), and ubiquitin (1–5). HSPs, as a group, are among the most abundant proteins in the cytosol and function in multicomponent complexes as molecular chaperones. HSP function is best understood under conditions of cellular stress (hypoxia, heat), during which levels of HSPs are dramatically increased (3, 6). Under these conditions, HSPs promote cell survival by preventing protein aggregation and promoting the refolding of damaged proteins (1–3, 7, 8).

In addition to having a key role in mitigating cellular stress, HSPs also play a critical role in unstructured cells. HSP70, and to a lesser extent HSP90, are critically involved in what are called “protein holding” functions (8–10). These HSPs form folding complexes that use ATP to help create the intramolecular interactions necessary for client protein folding (3, 11). The protein holding and folding functions of HSPs are critical in all normal cells, consistent with the embryonic lethal nature of HSP deletion (12).

The protein holding and protein folding functions of the HSPs are critical not only in normal unstressed cells, but also in transformed cells, in which the normal functions of HSPs are used to facilitate cell growth and cell cycle progression. As an example, HSP90 stabilizes Akt and oncopgenic forms of mutant epidermal growth factor receptor, both of which contribute to the growth of a variety of cancers including gliomas (13–15). HSP70, in a complex with HSP90 and HSP40, also serves to regulate the function and turnover of the estrogen receptor, increasing its activation and driving estrogen-dependent cellular responses (16, 17). HSP70 and HSP90 are also critically involved in the DNA-binding properties and stability of p53, and by stabilizing mutant p53, contribute to its role in transformation (18, 19). These observations suggest that the normal protein folding functions of HSPs, and in particular HSP70 and HSP90, are subverted by tumors to stabilize proteins critical for the establishment and maintenance of the transformed phenotype.

Although HSPs play a key role in the regulation of cell growth and cell cycle progression, their antiapoptotic properties may be even more important to the persistence of malignant cells. The intrinsic apoptotic pathway is driven by mitochondrial release of cytochrome <i>c</i> to the cytosol, which in turn triggers the polymerization of Apaf-1. Apaf-1 recruits procaspase-9 and procaspase-3 into the apoptosome, where the caspases are activated (20, 21). HSP70 reportedly interacts with Apaf-1, thereby preventing the interaction of Apaf-1 with procaspase-9 (22–24). Overexpression of HSP27 also increases the resistance of cells to various apoptotic stimuli reportedly by directly binding to the cytosolic cytochrome <i>c</i> and sequestering it from Apaf-1 (25, 26). In addition, HSP20 has been reported to complex with the proapoptotic protein Bax, which prevents the translocation of Bax from the cytosol into the mitochondria during apoptotic insult (27). As a result, HSP20 may preserve the integrity of mitochondria, restrict the release of cytochrome <i>c</i>, and repress the activation of caspase-3. The antiapoptotic effects of HSPs seem to be of particular importance in the cancer setting because targeted disruption of HSP27 or HSP70 leads to the activation of programmed cell death pathways (28). These results suggest that the ability of HSPs to block programmed cell death may help sustain the transformed cells and perhaps also to contribute to resistance to proapoptotic therapeutic agents.

Although HSP20, HSP27, and HSP70 are recognized as controllers of programmed cell death, the role of HSP90 in this process...
is less clear. There are two forms of HSP90 in the cytosol, HSP90α and HSP90β, which when combined, comprise ~1% to 2% of total cellular proteins (29, 30). Although these highly related isoforms differ in their inducibility (HSP90α is inducible, HSP90β is constitutively expressed), the functional differences between the two proteins are not well defined. Both contain a highly conserved ATP-binding domain near their NH2 terminus, and the chaperoning activity of both requires the binding of ATP at this site (31, 32). The middle region of both HSP90α and HSP90β has a key role in the binding of client proteins (33), whereas the COOH terminus domain facilitates the dimerization of HSP90 (34, 35). The COOH terminus domain of HSP90α also contains a conserved EVD motif that recruits various co-chaperones, such as the immunophilins and HSP70/HSP90-organizing protein, which, in turn, modify the specificity of the HSP90-containing complexes (34, 35). HSP90 has been reported to play a role in apoptosis induced by nicotinamide, and HSP90 also binds to a variety of clients, many of which play key roles in the control of apoptosis (14, 36–38). In particular, HSP90 has been reported to bind to FLIP, a protein that interacts with FADD and is a key suppressor of tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in gliomas (37, 39). Although the exact isoforms of HSP90 and FLIP involved in this interaction have not been defined, our interest in TRAIL as a targeted chemotherapeutic agent, and in understanding the pathways that control TRAIL sensitivity/resistance in gliomas, led us to examine the possibility that HSP90α, via its potential ability to bind to and stabilize FLIPα, might also be a key regulator of TRAIL-induced apoptosis. We here show that HSP90α does regulate TRAIL-induced apoptosis, but not by altering FLIPα stability. Rather, HSP90α interacts with FLIPα and inhibits its ability to localize to its site of action in the death-inducing signal complex. Furthermore, the ability of HSP90α to localize antiauxotrophic proteins to the death-inducing signaling complex (DISC) is not limited to FLIPα, but also applies to at least two other antiauxotrophic proteins, RIP and FLIPβ. These results identify a novel means of apoptotic control by a HSP, and also identify a novel mechanism by which HSP90α can control sensitivity to a variety of apoptotic stimuli including TRAIL.

Materials and Methods

Cell culture and drug treatment. Immortalized or Ras-transformed human astrocytes were generated and cultured as described previously (40). Human recombinant TRAIL was kindly provided by Avi Ashkenazi (Genentech, South San Francisco, CA). Cells were exposed to TRAIL (0 or 800 ng/ml, 24 h) and lyzed, after which the DISC-related proteins were immunoprecipitated using an anti-FADD antibody. For immunoprecipitation of HSP90α, FLIPα, FLIPβ, and RIP, cells were allowed to reach 70% confluency in DMEM. Following incubation with TRAIL (0 or 800 ng/ml) for 24 h, the cells were washed once with ice-cold PBS and harvested using ٠ ice-cold cell lysis buffer supplemented with 1 mmol/L of phenylmethylsulfonyl fluoride and incubated on ice for 10 min. HSP90α, FLIPα, FLIPβ, or RIP was selectively immunoprecipitated from 200 μg of protein (whole cell lysates) by combining the cell lysate with 20 μL of FADD antibody (Cell Signaling Technology) conjugated to agarose A/G beads (Santa Cruz Biotechnology) followed by gentle rotation for 4 h at 4°C. In some instances, an HSP90α antibody was used to immunoprecipitate FLIPα, FLIPβ, or RIP following incubation with TRAIL. In order to immunoprecipitate the various FLAG-tagged NH2- or COOH-terminal HSP90α deletion mutants, the cell lysate of NH2- or COOH-terminal HSP90α deletion mutant—expressing cells was combined with an antibody targeting the FLAG epitope (Cell Signaling Technology). Samples were then centrifuged briefly (30 s, 2,000 × g) and pellets were washed twice with ٠ lysis buffer. Immunocomplexes (pellets) were eluted in 3 × SDS sample buffer. The levels of HSP90α, FLIPα, FLIPβ, and RIP in the assembled DISC, as well as expression of the FLAG-tagged HSP90α NH2- or COOH-terminal deletion mutants, were assessed by Western blot analysis. Immunoprecipitations carried out using a non-specific normal mouse IgG antibody were included as negative controls, as were the analyses of cells to which TRAIL (800 ng/ml) was added following lysis.

Immunoblot analysis. Cells were washed with ice-cold PBS, scraped from the culture dish, and incubated in tissue lysis buffer containing 10 mmol/L of KCl, 1 mmol/L of sucrose, 2 mmol/L of MgCl2, 0.5% Igepal CA-630, 1 mmol/L of EDTA, 1 mmol/L of DTT, 10 mmol/L of β-glycerophosphate, 1 mmol/L of Na3VO4, 10 mmol/L of NaF, 100 μg/mL of phenylmethylsulfonyl fluoride, and 10 μg/mL of aprotinin (all reagents were purchased from Sigma) for 30 min on ice. The cell lysate was centrifuged, and the supernatant was stored at –80°C until use. The protein concentration of extracts was measured using Protein Assay reagent (Bio-Rad Laboratories). Protein (30 μg) was subjected to SDS-PAGE and electroblotted onto Immobilon-P membrane (Millipore). The membrane was blocked in 5% nonfat skin milk/TBST [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L of NaCl, 0.1% Tween 20] at 4°C overnight and incubated with rabbit polyclonal antibody against HSP90α, PHA, phosho-Akt, FLIPα, FADD, FLIPβ, TANK, RIP, TRAF, or α-tubulin (all antibodies obtained from Cell Signaling Technologies). Bound antibody was detected with antirabbit IgG (Santa Cruz Biotechnology) using enhanced chemiluminescence Western blotting detection regents (Amersham Pharmacia Biotech, Inc.). Densitometric measurements of immunoreactive bands were acquired using an Alphalager 2200 (Alpha Innotech Corporation). The expression of α-tubulin was used to verify equal loading.

Analysis of apoptosis by flow cytometry. For the analysis of apoptosis, cells were incubated with either TRAIL (800 ng/ml) or vehicle control, after which, cells were harvested and monitored for the percentage of cells with sub-G1 DNA content (apoptotic cells) using propidium iodide as previously described (41). For cell cycle analysis, harvested cells were centrifuged and resuspended in PBS after which, the cells were fixed by the addition of ice-cold 70% ethanol and stored at –20°C for 1 h. Subsequently, cells were pelleted by centrifugation and resuspended in staining solution containing 1 mg/mL of propidium iodide (Sigma) and 50 mg/mL of RNase A (Sigma) in PBS. After 5 min of incubation at 37°C, the cell suspension was passed through a 40-μm pore size cell strainer (Becton Dickinson) and subjected to flow
cytometry using a Becton Dickinson FACSort flow cytometer. The data was analyzed using CellQuest and ModFit software (Becton Dickinson). For the purpose of analysis, acquired events (20,000 cells) were gated to eliminate cell aggregates and debris.

Results

Because the protein stabilizing property of HSP90α has been linked to the suppression of apoptosis, we addressed the possible involvement of HSP90α in the control of TRAIL-induced apoptosis. We focused these studies on HSP90α because only HSP90α, and not HSP90β, was up-regulated in glioma cells.4 To begin these studies, normal human astrocytes immortalized and/or transformed by the expression of one of four different forms of V12 H-Ras (E6/E7/hTERT/Ras cells expressing V12 H-Ras, G37 cells expressing V12 H-Ras E37G, S35 cells expressing V12 H-Ras T35S, or C40 cells expressing V12 H-Ras Y40C) were obtained. These cells, which exhibit different levels of FLIP S expression and TRAIL sensitivity (E6/E7/hTERT/Ras and G37 have low levels of FLIPS and low TRAIL sensitivity; ref. 42) were first incubated with siRNA targeting HSP90α or a nonspecific scrambled siRNA, and analyzed for levels of HSP90α (A), HSP90β (B), phospho-Akt (C), and FLIP S (D) protein 24 to 72 h after siRNA addition. α-Tubulin was used as a loading control. FLIP S exposures in (A) are longer than those in (B) because of relatively low levels of FLIP S expression in the E6/E7/hTERT/Ras and G37 cells. The data are representative of one of three independent experiments for each group.

Figure 1. Effects of siRNA targeting HSP90α on HSP90α, HSP90β, phospho-Akt, and FLIP S protein levels. Normal human astrocytes were serially infected with retroviruses encoding E6, E7, hTERT, and either a constitutively active form of mutant V12 H-Ras (E6/E7/hTERT/Ras), or a mutant V12 H-Ras that was additionally modified to selectively activate the Raf (G37), Raf (S35), or phosphoinositide-3-kinase (C40) pathway. Cells were then mock transfected (CTRL), or transiently transfected with siRNA targeting HSP90α or a nonspecific scrambled siRNA, and analyzed for levels of HSP90α (A), HSP90β (B), phospho-Akt (C), and FLIP S (D) protein 24 to 72 h after siRNA addition. α-Tubulin was used as a loading control. FLIP S exposures in (A) are longer than those in (B) because of relatively low levels of FLIP S expression in the E6/E7/hTERT/Ras and G37 cells. The data are representative of one of three independent experiments for each group.

- Unpublished data.
FLIPS, we considered the possibility that HSP90α might control TRAIL sensitivity not by altering FLIPS levels, but rather by altering FLIPS localization. To begin to address this possibility, TRAIL-sensitive cells (E6/E7/hTERT/Ras, G37) with high levels of HSP90α and low levels of FLIPS were first retrofitically infected with a construct encoding cdc42 (a protein shown to translationally up-regulate FLIPS expression; ref. 42), after which levels of FLIPS, HSP90α, and TRAIL-induced apoptosis were examined in TRAIL-treated cells. Introduction of the cdc42-encoding construct had no effect on the levels of HSP90α protein, but did increase the levels of FLIPS protein relative to cells receiving empty vector (Fig. 3, bottom, lanes 3 and 4) and to cells receiving the cdc42-encoding construct but not exposed to TRAIL (42). Consistent with previous reports (42, 44), the cdc42-mediated increase in FLIPS protein levels decreased TRAIL sensitivity relative to cells that received only empty vector (Fig. 3A, lanes 3 and 4). The cdc42-overexpressing, FLIPS up-regulated E6/E7/hTERT/Ras and G37 cells were then incubated with an siRNA targeting HSP90α, after which levels of HSP90α and FLIPS, and the extent of TRAIL-induced apoptosis were assessed. Incubation of cdc42-overexpressing cells with siRNA targeting HSP90α effectively reduced the levels of HSP90α protein whereas having no effect on the levels of FLIPS protein relative to cells receiving scramble siRNA control (Fig. 3C, lanes 3 and 6); however, as in cells with endogenously high levels of FLIPS (S35, C40; Fig. 2), siRNA targeting HSP90α sensitized the FLIPS overexpressing E6/E7/hTERT/Ras and G37 cells to TRAIL-induced apoptosis relative to cells that received a scrambled siRNA control (Fig. 3A, lanes 5 and 6).

Having shown that siRNA-mediated suppression of HSP90α confers TRAIL sensitivity in cells endogenously or exogenously overexpressing FLIPS and that TRAIL-induced apoptosis was not associated with changes in absolute FLIPS levels, we directly examined the possibility that HSP90α enhances TRAIL-induced apoptosis by chaorpening FLIPS to its site of action in the DISC. To do so, lysates from TRAIL-incubated E6/E7/hTERT/Ras and G37 cells (low levels of FLIPS) or cdc42 overexpressing E6/E7/hTERT/Ras or G37 cells (high levels of FLIPS) were subjected to immunoprecipitation using an antibody targeting the DISC-related proteins FADD, after which protein levels of HSP90α and FLIPS were determined. The levels of FLIPS protein were then assessed by Western blotting using an antibody to FLAG (Fig. 4). Deletion of the COOH-terminal domain mutants, and the FLAG-tagged COOH-terminal domain deletion mutants were then verified by Western blot using an antibody to FLAG (Fig. 4). HSP90α protein levels were necessary for FLIPS binding and localization, and cells with high levels of FLIPS (C40 and S35) were first transiently transfected with various constructs encoding avian FLAG-tagged deletion mutants of HSP90α (the avian HSP90α is 94% homologous to the human protein; ref. 2, forms a functional complex with HSP90α co-chaperones; ref. 34, and is widely used to study HSP90α-client protein interactions). Expression of the control FLAG-tagged NH2-terminal domain mutants, and the FLAG-tagged COOH-terminal domain deletion mutants were then verified by Western blot using an antibody to FLAG (Fig. 4.4, S35; data not shown). Having established the expression of the FLAG-tagged HSP90α deletion mutants, the transfected C40 and S35 cells were lysed after which the lysate was subjected to immunoprecipitation using an antibody targeting the DISC-related proteins FADD, after which protein levels of HSP90α and FLIPS were monitored by Western blotting.

Figure 2. siRNA targeting HSP90α sensitizes otherwise TRAIL-resistant S35 and C40 cells to TRAIL-induced apoptosis without altering total FLIPS levels. TRAIL-sensitive E6/E7/hTERT/Ras and G37 cells (A) and TRAIL-resistant S35 and C40 cells (B) were exposed to TRAIL (0 or 800 ng/mL, 24 h), or incubated with either a scramble siRNA control or siRNA targeting HSP90α for 48 h followed by the addition of TRAIL for 24 h, stained with propidium iodide, and analyzed by flow cytometry for the percentage of cells having a <2N DNA content (apoptotic cells; top). An aliquot of cells from each group described was lysed (top), after which, levels of HSP90α and FLIPS were monitored by Western blot (bottom). α-Tubulin was used as a loading control. Both 1x (30 s) and 5x FLIPS exposures are provided for the E6/E7/hTERT/Ras and G37 cells, which express low levels of FLIPS relative to the C40 and S35 cells (1x exposure only). Top, columns, means; bars, SEs (n = 3). Bottom, Western blots were representative of one of three independent experiments for each group.

www.aacrjournals.org 9485 Cancer Res 2007; 67: (19). October 1, 2007

HSP90α targets TRAIL-induced FLIPS localization and TRAIL Sensitivity
The levels of a variety of DISC-related proteins were then assessed in cell lysates and in DISC complexes immunoprecipitated using a FADD-directed antibody. siRNA-mediated suppression of HSP90α levels did not alter the total cellular levels of any of the DISC-related proteins relative to controls (TRAIL alone, scrambled siRNA plus TRAIL, and lysates exposed to TRAIL; Western blot; Fig. 5). It did, as expected, significantly decrease levels of FLIP<sub>S</sub> in the immunoprecipitated DISC (Fig. 5, lanes 5 versus 4). siRNA-mediated suppression of HSP90α levels, however, also significantly decreased the levels of FLIP<sub>S</sub> and RIP in the DISC, whereas not altering levels of FADD, TANK, or TRAF. These results show that HSP90α serves as a selective chaperone for a subfamily of DISC-related proteins, and that this action, rather than protein stabilization or alteration of protein levels, could allow HSP90α to globally regulate apoptosis initiated by a variety of stimuli.

Having established that HSP90α interacts with the FLIP<sub>S</sub> protein, we considered the possibility that HSP90α may interact with a complex of DISC-related proteins, maintaining the stability of these client proteins prior to recruitment to the DISC, as previously suggested by Lewis et al. (45). To do so, C40 cells with high levels of FLIP<sub>S</sub> and HSP90α were lysed, after which, the proteins in the lysate were immunoprecipitated using an antibody targeting HSP90α. Following elution of the immunoprecipitated proteins, levels of HSP90α, FLIP<sub>S</sub>, FLIPL, RIP, FADD, TANK, and TRAF proteins were then monitored by Western blot and compared with total levels of these proteins from an aliquot of lysates obtained prior to immunoprecipitation. Although the C40 cells expressed high levels of all DISC-related proteins as assessed by Western blot prior to immunoprecipitation (Fig. 6B), only HSP90α, FLIP<sub>S</sub>, FLIPL, and RIP were present in the immunoprecipitated HSP90α protein complex (relative to proteins immunoprecipitated with an IgG antibody; Fig. 6A, lanes 1 and 2). Similar results were obtained using S35 cells (data not shown). These studies show that HSP90α, in a manner that is ATB-binding domain-dependent, interacts with a complex of DISC-related proteins consisting of FLIP<sub>S</sub>, FLIPL, and RIP in resting cells and allows the recruitment of these proteins to the DISC following apoptotic stimulation.

**Discussion**

The ability of HSP90α to bind and stabilize potentially oncogenic proteins has long been recognized as critical to the tumorigenic process. Accumulating evidence, however, suggests that HSP90α also contributes to tumorigenesis in other ways. The present study provides evidence that in addition to binding and stabilizing potentially oncogenic proteins, HSP90α helps localize FLIP<sub>S</sub> and a subset of other antiapoptotic proteins to their site of action in the DISC. The immediate consequence of this action is that HSP90α regulates TRAIL-induced apoptosis and has the potential to contribute to TRAIL resistance. In a broader sense, however, the novel localization function of HSP90α may help explain the global antiapoptotic functions of HSP90α and its overexpression in a variety of cancers.

Although our observation that HSP90α down-regulation decreased pAkt levels was expected, the inability of HSP90α down-regulation to alter FLIP<sub>S</sub> levels was initially surprising. Interaction of client proteins with HSPs typically results in increased client protein stability and increased client protein levels. In the present study, however, FLIP<sub>S</sub> levels were not altered by HSP90α suppression or pAkt suppression. This was particularly surprising because decreased levels of pAkt (as seen following exposure to HSP90α siRNA) have been reported to decrease translation of FLIP<sub>S</sub>.
importance of HSP90

antiapoptotic protein to its site of action in the DISC. Given the example of an HSP-client interaction leading to localization of an endoplasmic reticulum (48). The present study represents the first these complexes, resulting in both protein instability as well as (48). Treatment with an HSP90 inhibitor leads to the disruption of protein eluted from anti-FLAG antibody immunoprecipitates using an antibody targeting FLIP$_S$. To verify equal levels of FLIP$_S$ protein among the experimental groups, total levels of FLIP$_S$ protein in the lysates of the deletion mutant-expressing cells were determined by Western blot using an aliquot of lysate taken prior to immunoprecipitation. The data are representative of one of three independent experiments for each group.

mRNA and lower FLIP$_S$ protein levels (39). Although HSP90ox has the potential to regulate the Akt-mTOR-FLIP$_S$ pathway at multiple levels, the end result of suppression of HSP90ox was clearly not decreased levels of FLIP$_S$. FLIP$_S$, however, as previously reported (39, 42, 46), did play a key role in controlling TRAIL sensitivity as cdc42 overexpression (which drives FLIP$_S$ overexpression independently of Akt levels; refs. 42, 44) increased both FLIP$_S$ levels and TRAIL resistance (Fig. 3). These results, as a whole, suggest that the although in some cases HSP90ox serves to control the protein levels of client proteins, in other cases, it alters client protein function in more subtle ways.

The ability of HSP90ox to interact with FLIP$_S$, and guide it to the DISC, is both consistent with known HSP function and novel. HSPs typically interact with client proteins in a manner dependent on the NH$_2$-terminal ATP-binding domain of the HSP. The interaction between HSP90ox and FLIP$_S$ seems to follow this paradigm as the ATP-binding NH$_2$-terminal domain was required for the HSP90ox-FLIP$_S$ interaction. Although the interaction between HSPs and client proteins follows a standard route, the end result of HSP/client protein interactions can be variable. In some cases, the interaction could lead to stabilization and protection from degradation, whereas in other instances, intracellular targeting of the proteins occurs. As an example, HSP90 has been reported to stabilize RIP (ref. 45; although our studies with siRNA-targeting of HSP90ox, but not HSP90x). HSP90x has also been reported to protect the mutant form of p53 from degradation in some tumors (18, 19), or localize the active form of the estrogen receptor from the cytosol to the nucleus where it interacts with the estrogen response element (47). In some instances, HSP90x seems to be involved in both stabilizing and localizing client proteins. As an example, Her-2 (p185$^{erbB2}$), a receptor tyrosine kinase overexpressed in a significant proportion of malignancies, binds to HSP90x and the HSP90x endoplasmic reticulum homologue, Grp94 (48). Treatment with an HSP90 inhibitor leads to the disruption of these complexes, resulting in both protein instability as well as the inability of the nascent Her-2 peptide to localize to the endoplasmic reticulum (48). The present study represents the first example of an HSP-client interaction leading to localization of an antiapoptotic protein to its site of action in the DISC. Given the importance of HSP90x in the interaction, and that the FLIP$_S$/HSP90x complex is recruited to the DISC only following pro-apoptotic stimuli, the results suggest that DISC assembly creates or reveals a binding site for the HSP90ox-FLIP$_S$ complex through direct interaction with HSP90ox. HSP90x displays a binding preference for the oC$_{14}$ loop region found in a variety of client proteins (49). One possibility, therefore, is that this HSP90x-binding motif is revealed following DISC assembly in response to apoptotic

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**Figure 4.** The NH$_2$-terminal ATP-binding domain of HSP90ox is both necessary and sufficient to bind to FLIP$_S$. TRAIL-resistant C40 and S35 cells were transiently transfected with an empty vector (pFLAG-CMV 6c), or a vector encoding various NH$_2$-terminal or COOH-terminal deletion mutants of HSP90ox. A, expression of the deletion mutants was verified by Western blot analysis using an anti-FLAG antibody 48 h following transfection (S35 cells not shown). B, the interaction between the HSP90ox deletion mutants and FLIP$_S$ in C40 and S35 cells was assessed in protein eluted from anti-FLAG antibody immunoprecipitates using an antibody targeting FLIP$_S$. To verify equal levels of FLIP$_S$ protein among the experimental groups, total levels of FLIP$_S$ protein in the lysates of the deletion mutant-expressing cells were determined by Western blot using an aliquot of lysate taken prior to immunoprecipitation. The data are representative of one of three independent experiments for each group.

**Figure 5.** HSP90x serves as a chaperone for multiple DISC-related proteins. TRAIL-resistant C40 cells were either exposed to vehicle or TRAIL (800 ng/mL, 24 h), or were transfected with scrambled siRNA or siRNA targeting HSP90ox, after which the transfected cells were exposed to TRAIL (800 ng/mL) during the last 24 h of the 72-h siRNA incubation. Cells were then lysed and the levels of total or DISC-immunoprecipitated client proteins were determined by Western blot. Lane 2, total or DISC-immunoprecipitated proteins from lysate incubated with TRAIL (800 ng/mL, 24 h). α-Tubulin was used as a loading control. The data are representative of one of three independent experiments for each group.
also need HSP90, FLIPS, and that high levels of FLIPS confer TRAIL resistance (39). The activation of the Akt-mTOR pathway translationally up-regulates resistance in gliomas. Previous work from our lab showed that TRAIL resistance, and that in addition to high levels of FLIPS, cells with the DISC remains to be examined.

tumor cells (and all glioblastoma multiforme cells we have that high levels of FLIPS in the cytosol is not sufficient to confer one of three independent experiments for each group.

The ability of HSP to target FLIPS to the DISC in response to TRAIL seems to be critical for the key role HSP90α plays in TRAIL resistance in gliomas. Previous work from our lab showed that activation of the Akt-mTOR pathway translationally up-regulates FLIPS, and that high levels of FLIPS confer TRAIL resistance (39).

The present data, although consistent with this idea, adds a new layer of complexity to FLIPS regulation. The present work shows that high levels of FLIPS in the cytosol is not sufficient to confer TRAIL resistance, and that in addition to high levels of FLIPS, cells also need HSP90α to localize FLIPS appropriately. Because many tumor cells (and all glioblastoma multiforme cells we have examined) have elevated levels of HSP90α, low levels of HSP90α do not seem to be a cause of TRAIL resistance. In work in stimuli, although this possibility, and the site of HSP90α interaction with the DISC remains to be examined.

The ability of HSP to target FLIPS to the DISC in response to TRAIL sensitivity, the present work suggests that HSP90α alters the DISC localization of several antiapoptotic proteins, and by doing so, may affect apoptosis in a more global manner than that previously suspected. The present work shows that HSP90α interacts with at least three antiapoptotic proteins (FLIPS, FLIPL, and RIP) in untreated cells, and targets these proteins to the DISC in response to TRAIL. Although FLIPL and RIP have been excluded from playing a role in controlling TRAIL sensitivity in glioma cells (39), both proteins have been reported to play key roles in controlling the sensitivity of various cell types to a variety of other proapoptotic agents (51, 52). The ability of HSP90α to localize all three proteins (and potentially other DISC components), suggests that HSP90α may facilitate global inhibition of apoptosis. Consistent with this idea, HSP90 has been shown to interact with and stabilize RIP, and by doing so, to suppress tumor necrosis factor–induced apoptosis (45). The up-regulation of HSP90α noted in many cancers may in fact be a prerequisite for tumor formation, serving as part of a global response to suppress apoptosis stimulated by undoubtedly harsh tumor microenvironmental conditions. Although this idea has not formally been tested, the ability of HSP inhibitors to induce apoptosis in a variety of tumor types suggests that an understanding of how HSP90α contributes to protein localization may contribute to a better understanding of cellular transformation, and ultimately to better cancer therapies.

Acknowledgments

Received 2/4/2007; revised 5/14/2007; accepted 7/20/2007.

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References

13. HSP90α interacts with FLIPβ, FLIPγ, and RIP proteins in resting cells. C40 cells were lysed and the levels of HSP90α, FLIPβ, FLIPγ, RIP, FADD, TANK, and TRAF proteins were immunoprecipitated using either an IgG antibody or an antibody targeting HSP90α (A), or levels of total cellular HSP90α, FLIPβ, FLIPγ, RIP, FADD, TANK, and TRAF proteins (B) were determined by Western blot. α-Tubulin was used as a loading control. The data are representative of one of three independent experiments for each group.

Figure 6. HSP90α interacts with FLIPβ, FLIPγ, and RIP proteins in resting cells. C40 cells were lysed and the levels of HSP90α, FLIPβ, FLIPγ, RIP, FADD, TANK, and TRAF proteins were immunoprecipitated using either an IgG antibody or an antibody targeting HSP90α (A), or levels of total cellular HSP90α, FLIPβ, FLIPγ, RIP, FADD, TANK, and TRAF proteins (B) were determined by Western blot. α-Tubulin was used as a loading control. The data are representative of one of three independent experiments for each group.

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