CAIR-1/BAG-3 Abrogates Heat Shock Protein-70 Chaperone Complex-mediated Protein Degradation

ACCUMULATION OF POLY-UBIQUITINATED Hsp90 CLIENT PROTEINS*

Received for publication, September 20, 2002, and in revised form, May 1, 2003
Published, JBC Papers in Press, May 14, 2003, DOI 10.1074/jbc.M209682200

Howard Doong‡§, Kathryn Rizzo‡, Shengyun Fang‡, Vyta Kulpa‡, Allan M. Weissman¶, and Elise C. Kohn‡§

From the ¶Molecular Signaling Section, Laboratory of Pathology and §Regulation of Protein Function Laboratory, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892

† The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ To whom correspondence may be addressed: Molecular Signaling Section, Laboratory of Pathology, 10 Center Dr., MSC 1500, Bethesda, MD 20892-1500. Tel.: 301-492-2726; Fax: 301-480-5142; E-mail: ek.koh@nih.gov.

§ The abbreviations used are: Hsp, heat shock protein; GA, geldanamycin; FL, full-length CAIR-1; dBAG, BAG domain deleted CAIR-1; CREB, cAMP-response element-binding protein; PARP, poly-ADP ribose polymerase; GST, glutathione S-transferase; p-Akt, phospho-Akt; EGFR, epidermal growth factor receptor; CDK-4, cyclin-dependent kinase 4.

CAIR-1/BAG-3 are regulatory co-chaperones for heat shock protein (Hsp) 70. Hsp70 facilitates the removal of injured proteins by ubiquitin-mediated proteasomal degradation. This process can be driven by geldanamycin, an irreversible blocker of Hsp90. We hypothesize that CAIR-1/BAG-3 inhibits Hsp-mediated proteasomal degradation. Human breast cancer cells were engineered to overexpress either full-length CAIR-1 (FL), which binds Hsp70, or a BAG domain-deletion mutant (dBAG) that cannot bind Hsp70. FL overexpression prevented geldanamycin-mediated loss of total and phospho-Akt and other Hsp client proteins. dBAG provided no protection, indicating a requirement for Hsp70 binding. Ubiquitinated Akt accumulated in FL-expressing cells, mimicking the effect of lactacystin proteasomal inhibition, indicating that CAIR-1 inhibits proteasomal degradation distal to protein ubiquitination in a BAG domain-dependent manner. Protein protection in FL cells was generalizable to downstream Akt targets, GSK3β, P70S6 kinase, CREB, and other Hsp client proteins, including Raf-1, cyclin-dependent kinase 4, and epidermal growth factor receptor. These findings suggest that Hsp70 is a chaperone driving a multiprotein degradation complex and that the inhibitory co-chaperone CAIR-1 functions distal to client ubiquitination. Furthermore, poly-ubiquitination is not sufficient for efficient proteasomal targeting of Hsp client proteins.
GA remains. Overexpression of CAIR-1 mimics the effect of the proteasomal inhibitor lactacystin, preventing the loss of polyubiquitinated proteins, without altering the capacity of the cell for ubiquitination. Inhibition of proteasomal degradation, similar to Akt, was observed for other Hsp90 client proteins. This suggests the negative co-chaperone function of CAIR-1 is a generalizable function. Protection of ubiquitinated client proteins is dependent upon the presence of the BAG domain, placing Hsp70 in a central position in this multimolecular degradation complex. These findings provide a possible explanation for the anti-apoptotic effects of CAIR-1 and suggest that targeting CAIR-1/Hsp70 interactions may be a novel molecular target for intervention.

EXPERIMENTAL PROCEDURES

Materials—Anti-Hsp70 (catalog SPA-811), -Hsc-70 (catalog SPA-816), -Hsp90, and -ubiquitin antibodies and Hsp70 and Hsp90 proteins were purchased from StressGen (Victoria, British Columbia, Canada). Antibodies to Akt, phospho (p)-Akt, p-GSK-3β, P70S6 kinase, p-P70S6 kinase, cleaved caspase-3, CREB, and p-CREB were obtained from Cell Signaling (Beverly, MA). Antibodies to GSK-3β, EGFR, CDR-4, Raf-1, PARP, and tubulin were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal anti-His6

FIG. 1. CAIR-1 expression abrogates loss of p-Akt and Akt in GA-treated cells. A, MDA-435 cells were constructed to clonally express His6-tagged full-length (FL) or BAG deletion mutant (dBAG). The presence of expressed protein is shown by immunoprecipitation (IP) with anti-CAIR-1 and confirmation with anti-His6. IB, immunoblot. B, GA treatment induces Hsp70 production. Wild type MDA-435 cells treated with 2 μM GA over an 18-h time course were assessed for quantity of Hsc70, Hsp90, endogenous CAIR-1, and Hsp70. Only Hsp70 is induced under GA treatment conditions. C, expression of FL protects both p-Akt and Akt from GA-mediated loss. Cells were exposed to 2 μM GA for 18 h prior to lysis, and lysates were subjected to immunoblot as indicated. Transient transfection with FL and dBAG versus neo confirmed these findings.
CAIR-1 Inhibits Proteasomal Protein Degradation

antibody was obtained from Clontech (Palo Alto, CA). pGEX-4T3 was from Amersham Biosciences. The mammalian expression vector PCINeo was from Promega (Madison, WI). Secondary antibodies, protein A/G beads, and BCA protein quantitation kits were purchased from Pierce. LipofectAMINE PLUS and other molecular reagents were from Invitrogen. FuGENE 6 transfection reagent was from Roche Applied Science. All other products were of molecular or analytical grade. Geldanamycin (GA) was generously provided by the Developmental Therapeutics Program, NCI, National Institutes of Health.

Preparation of CAIR-1 Mutants and Stable Transfectants—The full-length human CAIR-1 (FL) and the BAG-domain deleted CAIR-1 (dBAG) sequences were generated by PCR from plasmid containing the full-length CAIR-1 message, previously verified and published sequence (26,27). Amplified sequence was confirmed prior to subcloning into pCIneo for stable transfection. The 50-amino acid BAG domain deletion mutant (dBAG; deleted amino acids 445–494) (14) was engineered with an novel internal HindIII site. Both FL and dBAG constructs are tagged with His6 at the N terminus. FL CAIR-1 was generated using primers 1 and 2 and dBAG by ligating the N terminus (primers 1 and 3; below) to the C terminus (primers 4 and 2). Primer 1, sense, 5'-GGT CCT CCA GCC ACC ATG CAT CAC CAT CAC AGC ATT GAT GTC CCA-3'; primer 2, antisense, 5'-GCC TTC AAA GTT GTC TAC AGC CTG-3'; primer 3 antisense, 5'-ACC CAC-3'; primer 4, sense, 5'-GGT ACC CAC-3'.

Stable clonal cell lines were established with G418 selection. Cells were maintained in G418 400 μg/ml for stable transfection. The 50-amino acid BAG domain deletion mutant (dBAG; deleted amino acids 445–494) (14) was engineered with an novel internal HindIII site. Both FL and dBAG constructs are tagged with His6 at the N terminus. FL CAIR-1 was generated using primers 1 and 2 and dBAG by ligating the N terminus (primers 1 and 3; below) to the C terminus (primers 4 and 2). Primer 1, sense, 5'-GGT CCT CCA GCC ACC ATG CAT CAC CAT CAC AGC ATT GAT GTC CCA-3'; primer 2, antisense, 5'-GCC TTC AAA GTT GTC TAC AGC CTG-3'; primer 3 antisense, 5'-ACC AAG CTT GCC ACC TAA GTT GCC TTA TGC TTC ACC-3'; primer 4, sense, 5'-GGT AAG CTT GAA CAG AAA GCC ATT GAT GTC CCA-3'.

Stable clonal cell lines were established with 418 selection. Cells were maintained in G418 400 μg/ml until split for treatment and lysis. Transient transfection was done to confirm the findings of the single cell clonal sublines. Briefly, MDA-435 cells were subjected to transfection using FuGENE 6 reagent according to the manufacturer’s instructions. At 24 h, cells were washed, incubated with 2 μg GA for 18 h, and then lysed for study. Cells were transfectected with His6-CAIR-1. CAIR-1 expression was confirmed by its His6 tag.

RESULTS

CAIR-1 Inhibits Proteasomal Protein Degradation

FIG. 2. CAIR-1 interacts selectively with Hsp70 through its BAG domain. A, the CAIR-1 BAG domain selectively pulls down Hsp70 but does not recognize Hsp90. Pull-down assays were done using the engineered GST-BAG domain. GST alone was used as the negative control. Assays used either MDA-435 cell lysate (left panel) or recombinant Hsp70 (upper right panel) or commercial purified Hsp90 (lower right panel). The presence of Hsp70 or Hsp90 in cell lysate is confirmed in the lysate immunoblot lane. GA exposure (2 μM, 18 h) induces Hsp70 production (Fig. 1), increased CAIR-1-Hsp70 complex is observed with GA exposure. B, the dBAG mutant cannot co-precipitate Hsp70 from lysate. Cells were lysed, and the lysate was subjected to immunoprecipitation (IP) with anti-His6 and blotted for bound Hsp70. A lysate immunoblot of FL alone is included as a control (far right lane) to confirm Hsp70. C, GA treatment increases binding of Hsp70 to CAIR-1. Wild type MDA-435 cells were treated with 2 μM GA for the indicated times, lysed, and subjected to co-immunoprecipitation. Commensurate with the increase in Hsp70 production (Fig. 1), increased CAIR-1-Hsp70 complex is observed with GA exposure. Hsp90 or Hsc70 (constitutive Hsp) protein (not shown) nor with 2 μM GA treatment of wild type cells for up to 18 h (Fig. 1B). In contrast, the previously reported GA-mediated induction of Hsp70 was confirmed. Stable transfectants were next subjected to GA treatment, resulting in loss of both p-Akt and total Akt in neo and dBAG cells (Fig. 1C). Cells overexpressing FL-CAIR-1 showed a marked attenuation of GA-mediated loss. Hsp90, the specific molecular target for GA (19, 20), has been proposed to be the primary chaperone for Hsp-mediated protein degradation (10). For this reason, the ability of CAIR-1 to bind to Hsp90 was assessed (Fig. 2). As no CAIR-1/Hsp90 binding was observed in immunoprecipitations of whole cell lysates (not shown), GST pull-down constructs were engineered and tested. The CAIR-1 BAG domain, when expressed as a GST fusion protein, selectively bound native (Fig. 2A, left panel) and recombinant Hsp70 (right panel) in pull-down experiments. It did not bind endogenous or purified Hsp90 (left and right panels, respectively; Fig. 2A, bottom). Furthermore, Hsp70 co-immunoprecipitated with FL but not dBAG from cell lysates, con-
FIG. 3. Akt shifts chaperone binding during GA exposure from Hsp90 to Hsp70. Neo or FL cells were subjected to a time course of GA exposure (2 μM), lysed, and lysates immunoprecipitated (IP) with anti-Akt. Parallel immunoprecipitations were blotted (IB) for Hsp90 (A) and Hsp70 (B; 1:40,000 primary with 1:100,000 anti-rabbit secondary antibody). A CAIR-1-independent shift of Akt client protein binding from Hsp90 to Hsp70 is shown. C, Akt/chaperone binding was quantitated and normalized against total immunoprecipitated Akt (not shown) to correct for Akt loss in the face of GA exposure. Akt binding to Hsp90 increased transiently to ~2-fold over control, whereas binding to Hsp70 increased over 20-fold (p = 0.024). D, FL cells had net increase in Akt/Hsp70 binding beginning after 6 h compared with neo controls (20- versus 12-fold, respectively, p < 0.0001). E, Akt does not bind to Hsc70. No co-immunoprecipitation of Akt to Hsc70 (1:10,000 primary with 1:50,000 anti-rabbit secondary antibody) was identified under control or geldanamycin-treated conditions. Binding of Akt to Hsp70 was confirmed.
firming the requirement for the BAG domain for association of CAIR-1 with Hsp70. Finally, commensurate with the increased quantity of Hsp70 with GA treatment (Fig. 1B), GA treatment also resulted in increased CAIR-1/Hsp70 immunocomplex formation (Fig. 2C). The dependence of the BAG domain, the site of interaction with Hsp70, and the lack of CAIR-1 binding to Hsp90 suggest a primary role for Hsp70 in the protection of Akt by CAIR-1.

Akt Shifts Chaperone Binding from Hsp90 to Hsp70 during GA Exposure—Hsp90 client proteins have been shown to be subjected to proteasomal degradation (22). We confirm Akt client binding to Hsp90 prior to and during early GA exposure (Fig. 3). Parallel time course experiments demonstrate that Akt shifts primary chaperone from Hsp90 to Hsp70 in the same time course where Hsp70 is induced by GA. A 20-fold increase in Akt-Hsp70 complex is seen in the face of a stable 1.5–2-fold increase in Akt/Hsp90, when corrected for the loss of Akt over time (p = 0.024, Mann-Whitney; Fig. 3C). FL cells accumulated more Akt-Hsp70 complex than neo control cells beginning 6 h into incubation with GA (12 versus >20-fold, p = <0.0001). This indicates that the chaperone shift of Akt from Hsp90 to Hsp70 proceeds also in the control setting where substantial loss of Akt is occurring. No co-precipitation of Hsp70 and Hps90 could be demonstrated, suggesting that there is a shuttling of the Akt client protein from Hsp90 to Hsp70. Furthermore, Akt does not bind to Hsc70 in control or geldanamycin-treated cells (Fig. 3E), and geldanamycin does not induce Hsc70 production (data not shown).

CAIR-1 Expression Prevents Degradation of Poly-ubiquitinated Akt—Current models for degradation of proteins chaperoned by the Hsp90 complex include poly-ubiquitination through Hsp70-associated ubiquitin ligases followed by proteasomal degradation (9). The effect of CAIR-1 expression on Akt ubiquitination in response to GA exposure was next evaluated (Fig. 4). No notable accumulation of poly-ubiquitinated forms of Akt were observed in the absence of proteasome inhibition or CAIR-1 overexpression in neo vector control cells (Fig. 4A, upper panel). In contrast, substantial accumulation of ubiquitinated Akt was observed in GA-treated cells expressing FL (Fig. 4A, lower panel). Fig. 4B shows dBAG cells have a wild type phenotype, no accumulation of poly-ubiquitinated Akt (Fig. 4B, lanes 3–7 compared with lanes 8 and 9, neo and FL, respectively). The loss in total Akt in neo and dBAG cells compared with FL transfectants is shown in the lower panel. No difference in total protein ubiquitination was seen in the three cell lines arguing that there was no fundamental difference in the general function of the ubiquitin system (data not shown). A quantitative comparison of ubiquitinated Akt in the three cells is shown in Fig. 4C. The increase in ubiquitinated forms is disproportionate to the greater total Akt level in GA-treated FL cells compared with those expressing either neo or dBAG. These data suggest that GA is stimulating Akt ubiquitination and that CAIR-1 is preventing subsequent proteasomal degradation of the ubiquitinated forms.

To evaluate this further, cells were treated with the irreversible proteasome inhibitor, lactacystin (27). Cells were pretreated with lactacystin, and exposure to lactacystin continued during the GA treatment. This resulted in the accumulation of ubiquitinated forms of Akt, as might be expected for a ubiquitination substrate, in response to GA in neo and dBAG cells as well as in those expressing FL (Fig. 5, A and B). A quantitation of replicate experiments is shown in Fig. 5C, demonstrating the similar accumulation of ubiquitinated Akt in neo, dBAG, and FL cells. Lactacystin prevented the GA-induced loss of Akt in neo and dBAG cells consistent with a proteasomal route of degradation. It is possible that CAIR-1 initiates its protection of client proteins prior to interaction with the proteasome or that it occurs within that macromolecular complex. No evidence was found by co-immunoprecipitation studies that either CAIR-1 or Hsp70 interact directly with the regulatory or cata-
CAIR-1 inhibits proteasomal protein degradation by preventing poly-ubiquitinated Akt from GA-mediated loss. Thus, CAIR-1 functions to prevent degradation of poly-ubiquitinated Akt in a BAG domain and Hsp70-dependent fashion.

Akt Is a Substrate for Ubiquitination in the Absence of GA—Akt has been described to be a caspase substrate (28). It has not been shown previously to be ubiquitinated or degraded by the proteasome under nonpharmacologic conditions. Cells were thus exposed to lactacystin in the absence of GA and Akt protein, and ubiquitination was assessed (Fig. 6). Cells were exposed to lactacystin for a total of 22 h to mimic the duration of exposure used in the GA-driven ubiquitination experiments in Figs. 4 and 5. No difference in relative amounts of poly-ubiquitination or total Akt was detected in neo, dBAG, and FL cells. When exposed to GA, degradation of Akt is driven in the neo and dBAG cells but is attenuated markedly by CAIR-1 expression in FL cells. These studies demonstrate that Akt is a substrate for ubiquitination under physiologic culture conditions.
CAIR-1 Protects Akt-mediated Phosphorylation of Akt Partner Proteins—Akt regulates the activation of multiple downstream partners (25). Protection of phosphorylation of the Akt downstream partners would be expected if the function of Akt was intact. Treatment with GA in neo control and dBAG cells resulted in loss of phosphorylated forms of the Akt downstream partner proteins glycogen synthase kinase-3β (GSK-3β), P70S6 kinase, and cAMP-response element-binding protein (CREB; Fig. 7). In contrast, phosphorylated forms were observed at basal or near basal levels in FL cells. Some loss of total GSK-3β and P70S6 kinase protein was observed in GA-treated cells, suggesting that these proteins may themselves be clients for Hsp90/Hsp70 chaperoned degradation. Consistent with this possibility, ubiquitinated GSK-3β and P70S6 kinase was detected in GA-treated FL cells but not in neo control cells (Fig. 7B). These data show that Akt function is maintained by overexpression of FL CAIR-1 in the GA-treated cells.

CAIR-1 Protects Other Hsp90 Client Proteins—The Hsp70-Hsp90 multimolecular chaperone complex is a general mechanism for signal pathway regulation through protein degradation. A number of Hsp90 client proteins have been identified (10). To confirm that the effect of CAIR-1 may be a general mechanism, not limited to the Akt pathway, other well described Hsp90 client proteins were examined. Raf-1, cyclin-dependent kinase 4 (CDK-4), and epidermal growth factor receptor (EGFR) have been reported to be degraded upon GA treatment (20, 22, 29, 30). Their sensitivity to GA was confirmed in neo control cells and also observed in dBAG-expressing cells (Fig. 8). Similar to the findings shown for Akt and its downstream pathway partners, protection from GA-driven degradation of all three client proteins tested was observed in FL cells. Commensurate with this is the accumulation of poly-ubiquitinated forms of each client protein in FL cells in the absence of lactacystin (Fig. 8B). Therefore, the protective effect of CAIR-1 on poly-ubiquitinated chaperone client proteins appears to be a general means by which the chaperone degradation pathway can be regulated.

Overexpression of CAIR-1 Is Not a Global Protectant for Cell Injury—We next examined the effect of CAIR-1 on heat shock and staurosporine in order to evaluate whether its protective effects are seen in other forms of injury. Heat shock was selected as it induces Hsp70 but has no effect on Hsp90 (31) and staurosporine because it prevents induction of Hsp70 in response to injury and does not involve Hsp90 (32, 33). Cells were exposed at 44 °C, returned to normal culture conditions for increasing periods of time, and then assayed for activation of caspase-3 and cleavage of PARP as molecular markers of injury (Fig. 9). Overexpression of CAIR-1 protects cells from heat shock injury. Neo control and dBAG cells had demonstrable evidence of heat shock injury as observed by a greater number of floating cells and by a lower net total cellular protein content in the cultures. This apoptotic injury was confirmed by the presence of cleaved caspase-3 and cleavage of PARP selectively in neo control and dBAG cells. All cells had induction of Hsp70 production by 6 h post-heat shock. In contrast, treatment with 4 μM staurosporine caused visible cell injury in all cells by 3 h into exposure. Expression of dBAG and FL CAIR-1 may sensitize the cell to injury, as cleavage of PARP and caspase-3 appears in those cells as early as 3 h of injury. Akt is uniformly reduced in neo, dBAG, and FL cells through the staurosporine exposure, and there is little if any effect on Hsp70 and Hsp90 quantities. No binding to Hsp70 of Akt was seen at early time points, before Akt degradation was complete. These data suggest that CAIR-1 may be selective to forms of injury in which Hsp70 is induced and/or involved.

Discussion

BAG family proteins bind to and regulate Hsp70 function in protein folding and activation (4, 7, 34). Hsp70-chaperoned degradation protein clients are subject to ubiquitin-mediated proteasomal degradation. Chaperoned proteasomal degradation involves a multimolecular complex including Hsps70 and 90 and P60HOP in the chaperone component, a client protein, and access to the ubiquitination system and the proteasome (11, 12, 21). How and where the BAG proteins fit into this complex and what regulatory role they have has been unknown. We demonstrate that overexpression of CAIR-1 results in inhibition of the degradation of poly-ubiquitinated heat shock client proteins. It is a generalizable function under the geldanamycin exposure, neither client- nor pathway-specific. Furthermore, protection of the index client protein, Akt, resulted in protection of its function as shown by downstream pathway partner phosphorylation. The CAIR-1 BAG domain is required for this protection, indicating that the inhibitory action occurs through BAG domain-dependent binding to Hsp70. This is similar to the reported BAG domain-dependent inhibition of Hsp70 protein folding shown for BAG-1 and BAG-3 (4, 35). The abrogation of client protein degradation by CAIR-1 binding to Hsp70 indicates that Hsp70 has a prominent role in the degradation chaperone complex. This role for Hsp70 is underscored by the demonstration of the shift in chaperone protein binding of the Akt client protein from Hsp90 to Hsp70 occurring during the time course of poly-ubiquitination and subsequent protein degradation. The protective effect of CAIR-1 occurs late in the process leading to protein degradation, after poly-ubiquitination but prior to proteasomal degradation. A schematic model demonstrating how CAIR-1 might function in this manner is presented in Fig. 10. Thus, CAIR-1 plays an inhibitory function in post-ubiquitination proteasomal degradation of Hsp70 client proteins.

The findings that CAIR-1 can modulate outcome of poly-ubiquitinated proteins further supports a previously unappreciated degree of complexity in proteasomal targeting through Hsp70, suggesting that CAIR-1 and possibly other members of this family disrupt proteasomal signaling. The disruption occurs distal to protein ubiquitination and is in accord with accumulating evidence (36, 37) that poly-ubiquitination itself may not be sufficient to trigger proteasomal degradation. Interestingly, another chaperone protein involved in regulation of the outcome of poly-ubiquitinated proteins, the valosin-containing protein (VCP)/p97, is also an ATPase. Loss of VCP/p97
function is associated with an inhibition of ubiquitin-proteasome-mediated degradation and accumulation of ubiquitinated proteins (37). The abrogation of protein degradation may provide a possible explanation for the anti-apoptotic effects of CAIR-1 that have been described. Furthermore, data suggest that the native function of CAIR-1 may be to regulate signal protein quantity and function under basal and stressed conditions. Finally, they suggest that targeting CAIR-1 interactions may have utility as a pro-apoptotic intervention in treatment of malignancy and other pathology because of dysregulated signaling events. The demonstration that overexpression of CAIR-1 had protective effects against apoptosis due to heat shock, but not in neo controls or dBAG cells.

**Fig. 7. Protection of Akt results in maintenance of downstream pathway activation and reduced poly-ubiquitination of Akt partner proteins.** Cells were incubated with 2 μM GA for 18 h as indicated. A, maintenance of phosphorylation of downstream Akt effector partners in the presence of CAIR-1. p-GSK-3β, p-P70S6 kinase, and p-CREB was protected in FL cells but not in neo controls or dBAG cells. A GA-mediated reduction in total GSK-3β and p70S6 kinase is reversed by FL transfection. B, accumulation of poly-ubiquitinated GSK-3β and P70S6 kinase in FL cells. Lysates from GA-treated cells were subjected to analysis for poly-ubiquitinated GSK-3β and P70S6 kinase under the same conditions used to evaluate Akt poly-ubiquitination. IP, immunoprecipitation; IB, immunoblot; Ub, ubiquitin.

**Fig. 8. CAIR-1 protects multiple Hsp90 client proteins.** A, the protective effect of CAIR-1 is generalizable to other Hsp90 client proteins. Relative quantity of the well described Hsp90 client proteins, CDK-4, Raf-1, and EGFR, was evaluated in lysates from cells exposed to 2 μM GA for 18 h. FL expression protects all clients, whereas dBAG is ineffective. B, FL expression results in accumulation of poly-ubiquitinated client protein. GA-treated cells were lysed and subjected to analysis for poly-ubiquitinated client protein as described for Akt. Accumulation of client protein is observed only in the FL transfectants. IP, immunoprecipitation; IB, immunoblot; Ub, ubiquitin.

CAIR-1 Inhibits Proteosomal Protein Degradation
CAIR-1 overexpression protects cells from heat shock injury but not from staurosporine. 

**A.** CAIR-1 protects cells from heat shock. FL, dBAG, and neo control cells were exposed to heat shock (44 °C for 40 min) and then returned to culture conditions for the indicated hours. Cell lysates were examined for the presence of cleaved caspase-3, PARP cleavage products, induction of Hsp70, and expression of the His-tagged CAIR-1 or dBAG proteins in respective blots. dBAG cells and neo control cells underwent apoptosis in response to heat shock injury as demonstrated by the production of cleaved caspase-3 and PARP cleavage. No significant difference was seen in the ability of heat shock to induce Hsp70 production in neo, dBAG, or FL cells. Neo control, lanes 1, 4, 7, and 10; dBAG, lanes 2, 5, 8, and 11; FL, 3, 6, 9, and 12. B. Akt complexes with Hsp-70 after heat shock. Cells exposed to heat shock and then allowed to recover for 18 h were lysed, and lysates subjected to immunoprecipitation (IP) for Akt. IB, immunoblot. Akt is bound to Hsp-70 only in FL heat-shocked cells. C, no protection from staurosporine exposure. Cells were exposed to staurosporine 4 μM for up to 12 h as indicated, lysed, and lysates analyzed. Progressive PARP cleavage with commensurate loss of total PARP is seen in all cells, starting as early as 3 h in the dBAG and FL cells. A progressive increase in caspase-3 cleavage is seen. Loss of Akt progressed equally in all cell types in the absence of notable Hsp-70 and Hsp-90. Neo control, lanes 1, 4, 7, and 10; dBAG, lanes 2, 5, 8, and 11; FL, lanes 3, 6, 9, and 12. D, Akt/Hsp70 binding is not detectable in staurosporine-treated cells. Co-precipitation of Akt and Hsp70 was done to evaluate complex formation under the stress of staurosporine. Control (lanes 1–3), 3- (lanes 4–6), and 6-h (lanes 7–9) exposures were tested without demonstration of the immunocomplex. GA-treated neo (lane 11) controls confirm that the Akt-Hsp70 complex forms in cells treated with GA. Neo control, lanes 1, 4, 7, and 11; dBAG, lanes 2, 5, and 8; FL, lanes 3, 6, and 9.
with heat shock, also an inductor of Hsp70 but not staurosporine which blocks Hsp70 transcription, supports this conclusion. The BAG domain requirement and lack of direct interaction of CAIR-1 with Hsp90 suggests that this is a loosely held Hsp70-Hsp90 multiprotein complex or that Hsp90 may initiate the complex and focus the client protein down the pathway to Hsp70, where it is subsequently ubiquitinated and degraded.

BAG domain-containing proteins have been shown to promote or be a marker of malignant behaviors including proliferation (15, 39), experimental metastasis (40, 41), inhibition of apoptosis (8, 16, 17, 42), and to be associated with a poor outcome in breast cancer (43–45). Overexpression of CAIR-1 may both protect cells and support transformed cells through its inhibition of protein degradation. The removal of client proteins by ubiquitination and proteasomal degradation is a potential mechanism of action for the demonstrated BAG protein enhancement of cell survival, proliferation, and transforming activities in cancer (15, 17, 39, 42, 46, 47), and is thus a logical target for molecular therapeutics. We demonstrate protection of proteins key in proliferation and survival pathways in cancer cells, including Akt and its downstream substrates (24), and EGFR, CDK-4, and Raf-1. The protection of Akt has broader implications in that we now show that ubiquitination and proteasomal degradation is a physiologic mechanism for removal of Akt. Therapeutic modulation of the inhibitory activity of CAIR-1 may interrupt the Akt survival pathway under selected stresses. CAIR-1 has been shown to function as an anti-apoptotic protein in heat shock (Fig. 9) and in a BAX-driven background (8) but is not a general protectant as shown by the sensitivity of the FL cells to staurosporine. In the BAX studies, transient expression of CAIR-1 on the BAX background was sufficient to reduce apoptosis in a fashion similar to that seen with co-expression of BAX and BCL-2. Co-expression of sub-threshold quantities of BCL-2 and CAIR-1 in that study similarly abrogated BAX-induced apoptosis, showing additivity of these two proteins. We and others (5, 8) have shown that CAIR-1 and BCL-2 are binding partners, which may account for the synergism between CAIR-1 and BCL-2 shown in the BAX model. It also has been reported that CAIR-1 provides a survival signal in serum-deprived cells (42).

The data presented herein coupled with the heat shock and BAX/BCL-2 results argue that CAIR-1 may function in more than one pathway to regulate cellular response to pro-apoptotic events. The lack of effect in the staurosporine-treated cells shows the selectivity of the CAIR-1 activity to the Hsp70-associated pathways and indicates that it is not a global protector. The degradation promoting tool used in this study, GA, is now in clinical trials as an anti-cancer agent. The demonstrated blockade of GA-mediated protein degradation identifies CAIR-1 as a putative chemoresistance protein that may block the anti-cancer effect of GA in vivo. For these collective reasons, CAIR-1 is a logical molecular target to interrupt for cancer treatment.

**Acknowledgments**—We thank A. Vrailas for technical assistance and Drs. L. Liotta and G. Mills for suggestions and critical review.

**REFERENCES**

1. McClellan, A. J., and Frydman, J. (2001) *Nat. Cell Biol.* 3, E1–E3
2. Morimoto, R. I., and Santoro, M. G. (1998) *Nat. Biotechnol.* 16, 833–838
3. Bimston, D., Song, J., Winchett, D., Takayama, S., Reed, J. C., and Morimoto, R. I. (1998) *EMBO J.* 17, 6871–6878
4. Nollen, E. A. A., Kabakov, A. E., Brunsting, J. F., Kanon, B., Hohfeld, J., and Kampinga, H. H. (2001) *J. Biol. Chem.* 276, 4677–4682
5. Takayama, S., Xie, Z., and Reed, J. C. (1999) *J. Biol. Chem.* 274, 781–786
6. Gassler, C. S., Wiederkar, T., Brehmer, D., Bukau, B., and Mayer, M. P. (2001) *J. Biol. Chem.* 276, 32538–32544
7. Takayama, S., and Reed, J. C. (2001) *Nat. Cell Biol.* 3, 237–241
8. Lee, J., Takahashi, T., Yasuhara, N., Inazawa, J., Kamada, S., and Tsujimoto, Y. (1999) *Oncogene* 18, 6183–6190
9. Minnaugh, E., Chavany, C., and Neelers, L. (1996) *J. Biol. Chem.* 271, 22796–22801
10. Neelers, L., Schulte, T. W., and Minnaugh, E. (1999) *Invest. New Drugs* 17, 361–373
11. Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L. Y., and Patterson, C. (1999) *Mol. Cell. Biol.* 19, 4535–4545
12. Demand, J., Alberti, S., Patterson, C., and Hohfeld, J. (2001) *Curr. Biol.* 11, 1569–1577
13. Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Hohfeld, J., and Patterson, C. (2001) *Nat. Cell Biol.* 3, 93–96
14. Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) *Cell* 80, 279–284
15. Kudoh, M., Knee, D. A., Takayama, S., and Reed, J. C. (2002) *Cancer Res.* 62, 1904–1909
16. Liu, R., Takayama, S., Zheng, Y., Frosch, B., Chen, G., Zhang, X., Reed, J. C., and Zhang, X. (1999) *J. Biol. Chem.* 274, 16988–16992
17. Blasielli, A., Longatti, P., Alberi, D., Geraghty, S., Schneider, C., Ponzetto, C., and Comoglio, P. M. (1996) *EMBO J.* 15, 6205–6212
18. Romano, M. F., Festa, M., Pagliuca, G., Lerose, R., Bisogni, R., Chiurazzi, F., Storti, G., Volpe, S., Venuta, S., Turco, M. C., and Leone, A. (2003) *Cell Death Differ.* 10, 383–385
19. Grenert, J. P., Sullivan, W. P., Padden, P., Haystead, T. A. J., Clark, J., Minnaugh, E., Krutzsch, H., Ochel, H.-J., Schulte, T. W., Sausville, E.,

---

2 E. C. Kohn and H. Doong, unpublished results.
3 H. Doong and E. C. Kohn, unpublished data.
Neckers, L. M., and Toft, D. O. (1997) J. Biol. Chem. 272, 23843–23850.

20. Schulte, T. W., Blagosklonny, M. V., Ingui, C., and Neckers, L. (1995) J. Biol. Chem. 270, 24585–24588.

21. Jiang, J., Ballinger, C. A., Wu, Y., Dai, Q., Cyr, D. M., Hoefeld, J., and Patterson, C. (2001) J. Biol. Chem. 276, 42938–42944.

22. Basso, A. D., Solit, D. B., Munster, P. N., and Rosen, N. (2002) Oncogene 21, 1159–1166.

23. Sato, S., Fujita, N., and Tsuruo, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10832–10837.

24. Testa, J. R., and Bellacosa, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10983–10985.

25. Hemmings, B. A. (1997) Science 275, 628–630.

26. Doong, H., Price, J., Kim, Y. S., Gasbarre, C., Probst, J., Liotta, L. A., Blanchette, J., Rizzo, K., and Kohn, E. (2000) Oncogene 19, 4385–4395.

27. Lee, D. H., and Goldberg, A. L. (1998) Trends Cell Biol. 8, 397–403.

28. Widmann, C., Gibson, S., and Johnson, G. L. (1998) J. Biol. Chem. 273, 7141–7147.

29. Supino-Rosin, L., Yoshimura, A., Yarden, Y., Elazar, Z., and Neumann, D. (2000) J. Biol. Chem. 275, 21850–21855.

30. Schulte, T. W., and Neckers, L. M. (1998) Cancer Chemother. Pharmacol. 42, 273–279.

31. Bang, O.-S., Ha, B. Y., Park, E. K., and Kang, S.-S. (2000) Biochem. Biophys. Res. Commun. 278, 306–311.

32. Kim, S. H., Kim, J. H., Erdos, G., and Lee, Y. J. (1993) Biochem. Biophys. Res. Commun. 183, 759–763.

33. Erdos, G., and Lee, Y. L. (1994) Biochem. Biophys. Res. Commun. 202, 476–483.

34. Kanelakis, K. C., Morishima, Y., Dittmar, K. D., Galigniana, M. D., Takayama, S., Reed, J. C., and Pratt, W. R. (1999) J. Biol. Chem. 274, 34134–34140.

35. Takayama, S., Bimston, D. N., Matsuzawa, S., Freeman, B. C., Aime-Sempe, C., Xie, Z., Morimoto, R. I., and Reed, J. C. (1997) EMBO J. 16, 4887–4896.

36. Wang, Q., Song, C., and Li, C.-C. (2003) Biochem. Biophys. Res. Commun. 300, 253–260.

37. Dai, R. M., and Li, C.-C. (2001) Nat. Cell Biol. 3, 740–744.

38. Froesch, B. A., Takayama, S., and Reed, J. C. (1998) J. Biol. Chem. 273, 11660–11666.

39. Song, J., Takeda, M., and Morimoto, R. I. (2001) Nat. Cell Biol. 3, 276–282.

40. Yawata, A., Adachi, M., Okuda, H., Naishiro, Y., Takamura, T., Hareyama, M., Takayama, S., Reed, J. C., and Imai, K. (1998) Oncogene 16, 2681–2686.

41. Takaoka, A., Adachi, M., Okuda, H., Sato, S., Yawata, A., Hinoda, Y., Takayama, S., Reed, J. C., and Imai, K. (1997) Oncogene 14, 2971–2977.

42. Antoku, K., Maser, R. S., Scully, J. W. A., Delach, S. M., and Johnson, D. E. (2001) Biochem. Biophys. Res. Commun. 286, 1003–1010.

43. Tang, S., Shahtet, A., Chen, X., and Wang, X. (1999) J. Clin. Oncol. 17, 1710–1719.

44. Thress, K., Henzel, W., Shillinglaw, W., and Kornbluth, S. (1998) EMBO J. 17, 6135–6143.

45. Brimmell, M., Burns, J. S., Munson, P., McDonald, L., O’Hare, M. J., Lakhani, S. R., and Packham, G. (1999) Br. J. Cancer 81, 1042–1051.

46. Naishiro, Y., Adachi, M., Okuda, H., Yawata, A., Mitaka, T., Takayama, S., Reed, J. C., Hinoda, Y., and Imai, K. (1999) Oncogene 18, 3244–3251.

47. Liao, Q., Ozawa, F., Friess, H., Zimmermann, A., Takayama, S., Reed, J. C., Keleff, J., and Buchler, M. W. (2001) FEBS Lett. 503, 151–157.
CAIR-1/BAG-3 Abrogates Heat Shock Protein-70 Chaperone Complex-mediated Protein Degradation: ACCUMULATION OF POLY-UBIQUITINATED Hsp90 CLIENT PROTEINS
Howard Doong, Kathryn Rizzo, Shengyun Fang, Vyta Kulpa, Allan M. Weissman and Elise C. Kohn

J. Biol. Chem. 2003, 278:28490-28500.
doi: 10.1074/jbc.M209682200 originally published online May 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M209682200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 21 of which can be accessed free at http://www.jbc.org/content/278/31/28490.full.html#ref-list-1