HSP70 Protein Promotes Survival of C6 and U87 Glioma Cells by Inhibition of ATF5 Degradation

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The activating transcription factor 5 (ATF5, also known as ATFx) is a member of the ATF/cAMP response element-binding protein family of basic zipper proteins (12). ATF5 is expressed in a number of cancer cells and down-regulated in their respective normal cells (13–15). Notably, interference of ATF5 function in non-neoplastic breast cells or in non-tumor brain cells did not affect their survival (14, 15). Such selective survival dependence on ATF5 by different types of cells seems to result from the differential ability of ATF5 to regulate its downstream targets in different cells. In congruity, we recently identified ATF5 regulatory elements (ARE) in the promoter regions of both Bcl-2 and Egr-1 and showed that ATF5 regulates the expression of Bcl-2 and Egr-1 in an ARE-dependent manner in transformed cells such as C6 and MCF-7 cells but not in non-transformed cells such as rat astrocytes and human breast epithelial cells (16, 17).

Although both the heat shock protein 70 (HSP70) and the activating transcription factor 5 (ATF5) have been shown to promote cell survival of transformed cells but not survival of non-transformed cells, the relationship of the two molecules is unknown. Here we show that HSP70 and ATF5 are concomitantly up-regulated upon transient but down-regulated upon prolonged cellular stress and apoptotic stimulation in the rat C6 glioma and human U87 glioma cells. HSP70 interacts strongly with the N-terminal activation domain of ATF5, which is expected to be rigid and uniquely structured under physiological conditions because of extraordinary high concentration (over 25%) of proline residues. Binding of HSP70 to ATF5 is an ATP-driven process and requires functional ATPase on the nucleotide binding domain of the HSP70 molecule. Overexpression of HSP70 dramatically stabilizes the ATF5 protein, which is otherwise subject to rapid degradation, facilitated by both proteasome-dependent and caspase-dependent processes, whereas HSP70 depletion leads to acceleration of ATF5 degradation and transcription repression of Bcl-2 and Egr-1, which are downstream targets of ATF5 in C6 and U87 glioma cells. Our data reveal an essential role for HSP70 in maintaining high levels of ATF5 expression in glioma cells and support the conclusion that ATF5 is an important substrate protein of HSP70 that mediates HSP70-promoted cell survival in glioma cells.

Glioblastoma is the most common form of primary brain tumor that responds poorly to conventional therapy. The average survival time from diagnosis is less than 1 year (1, 2). These tumors, arising either from astrocytes or their progenitor cells, escape most normal cell growth and cell death control mechanisms (3).

The stress-inducible HSP70(2) (also known as HSP72 or iHSP70) is a molecular chaperone that plays an essential role in protein folding, stability, and turnover. HSP70 is highly expressed in malignant tumors of various origins, and its expression correlates with increased cell proliferation, poor differentiation, lymph node metastases, and poor therapeutic outcome in human cancer (4–7). In support of its role in promoting tumorigenesis via its prosurvival function, overexpression of HSP70 effectively inhibits cell death induced by a wide range of stimuli, including several cancer-related stresses like hypoxia, inflammatory cytokines, monocytes, irradiation, oxidative stress, and anticancer drugs. Indicating a cancer-specific cell survival function of HSP70, depletion of HSP70 induces massive apoptotic death in tumorigenic cells but not in non-tumorigenic epithelial cells or embryonic fibroblasts (8, 9). These lines of evidence suggest that HSP70 is required for protecting certain vulnerable proteins whose function is essential and specific for the survival of cancer cells. The HSP70 molecule, as all other members of the HSP70 family, has a modular structure that consists of an N-terminal nucleotide binding domain and a C-terminal substrate binding domain, connected by a short linker (10). The function of HSP70 family members appears to revolve around cross-talk between ATPase activity in the nucleotide binding domain and substrate binding in the substrate binding domain. Thus, mutants of HSP70 or other family members such as the glucose-regulated protein 94 (GRP94) defective in ATP hydrolysis are completely deficient in chaperone activities and unable to support cell survival (11). Although specific substrate proteins of HSP70 are expected and should be uniquely required for survival of transformed cells, the identity of these target proteins remains elusive.

The activating transcription factor 5 (ATF5, also known as ATFx) is a member of the ATF/cAMP response element-binding protein family of basic zipper proteins (12). ATF5 is expressed in a number of cancer cells and down-regulated in those cells following growth factor deprivation that leads to cell death. Overexpression of ATF5 suppresses apoptosis in HeLa cells induced by serum withdrawal and in FL5.12 cells, an IL-3-dependent cell line, from IL-3 deprivation (13). Conversely, ATF5 loss of function induces apoptosis of HeLa, FL5.12, and a number of glioma and breast cancer cell lines cultured in the presence of growth factors (13–15). Notably, interference of ATF5 function in non-neoplastic breast cells or in non-tumor brain cells did not affect their survival (14, 15). Such selective survival dependence on ATF5 by different types of cells seems to result from the differential ability of ATF5 to regulate its downstream targets in different cells. In congruity, we recently identified ATF5 regulatory elements (ARE) in the promoter regions of both Bcl-2 and Egr-1 and showed that ATF5 regulates the expression of Bcl-2 and Egr-1 in an ARE-dependent manner in transformed cells such as C6 and MCF-7 cells but not in non-transformed cells such as rat astrocytes and human breast epithelial cells (16, 17).
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ATF5 is known to subject to multilayered regulation that includes transcriptional regulation by early B-cell factor 1 EBF1 (18), translational regulation that is controlled by phosphorylated eukaryotic initiation factor 2 eIF2 (19, 20), and posttranslational regulation that may involve phosphorylation (21), acetylation (unpublished data)\(^3\), and ccdc34-dependent ubiquitin-mediated proteolysis (22–24). The N-terminal region of ATF5 molecule is Pro-rich, with more than 25% of amino acid residues being prolines, and is expected to be both rigid and uniquely structured, whereas the C-terminal region of ATF5 contains a basic zipper domain that may only be partially structured in solution under physiological conditions (25). Thus, additional folding instructions seem to be needed for ATF5 to form productive secondary and/or tertiary structures. In fact, to overcome the intrinsic instability of the ATF5 protein, either to preserve ATF5 from degradation/aggregation or to maintain correct confirmation, could be an essential layer of regulation in ATF5-promoted cell survival function. In this study, we tested the hypothesis that HSP70 promotes cancer cell survival by increasing the stability of ATF5. We provide evidence that HSP70 and ATF5 are concomitantly up- and down-regulated in C6 and U87 cells subject to various stress and apoptotic stimulations. HSP70 directly binds to ATF5 at its N-terminal region and dramatically increases ATF5 stability by inhibition of both proteasome-dependent and caspase-dependent degradation of ATF5. Our data support the conclusion that ATF5 is an important substrate protein of HSP70 and that ATF5 mediates HSP70-promoted cell survival in glioma cells.

MATERIALS AND METHODS

Plasmids—DNA constructs pCIN4 and pCIN4-FLAG-HA-ATF5, pLeGFP-C1-FLAG-ATF5 and pLeGFP-C1-FLAG-dnATF5, pEFGFP-FLAG-ATF5 and pQsiren-shRNA-ATF5, and HSP70- HA and HSP70(71E)-HA were described previously (17, 26, 27). To create pQsiren-shRNA-HSP70, 5′-GATCCGACCAAGATGGAAGATCCTCAAGAGATCCTCCTCATCTTGTTTCCTTACCGTG-3′ and 5′-AATTCCGCCTTTAAAGACCGCCAGATGGAGATCCTCCTTAGGATCTCCTCTTCTGTTGC-3′ were annealed to make a double-strand DNA fragment that was then cloned into RNAi-Ready pSIREN-RetroQ-ZsGreen between the BamHI and EcoRI sites to make shRNA-HSP70 against rat HSP70 mRNA.

Cell Culture, Serum Deprivation, and Drug Treatment—Rat C6 glioma and human U87 glioma cells were grown in DMEM (Invitrogen) with 10% FBS (Atlanta Biologicals), 100 μg/ml streptomycin, and 100 IU/ml penicillin. Stable cell lines C6-pCIN6 and C6-FLAG-HA-ATF5 were selected and maintained in DMEM with 10% FBS containing 800 μg/ml G418 (Clontech). For serum deprivation, cells (24 h after transfection if transfected cells were used) were washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM NaHPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\) (pH7.4)) and fed with DMEM without serum. For various drug treatments, stock solutions of arsenite trioxide (ARS, Sigma), thapsigargin (Tg, Sigma), camptothecin (cpt) (Sigma), MG132 (Calbiochem), boc-aspartyl(Ome)-fluoromethylketone (BAF, Enzyme Systems Products), cycloheximide (CHX, Sigma), and quercetin (Sigma) were added directly to the cell culture (24 h after transfection if transfected cells were used) to a final concentration as specified. Cell transfection was carried out using FuGENE 6 reagent (Roche) according to the manufacturer’s instructions.

Immunoblotting and Immunoprecipitation Analyses—Cells grown on culture plates were rinsed with ice-cold PBS and then lysed in cell lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% Triton X-100) containing a protease inhibitor mixture (Roche). The lysate was kept on ice for 30 min and then centrifuged at 12,000 × g for 10 min at 4 °C. For immunoblotting analysis, the supernatant was boiled with Laemmli sample buffer (63 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.0025% bromphenol blue (pH 6.8)), and was loaded on to an SDS-polyacrylamide gel. For immunoprecipitation analysis, we used 20 μl of protein A/G-agarose beads (Roche), 2 μg of antibody, and 1 mg of protein extract, following the manufacturers protocol. The antibodies used for immunoblotting analysis were rabbit anti-ATF5 (1:250) and mouse anti-β-actin (1:10,000) from Abcam; mouse anti-FLAG (1:1000) from Stratagene; rat anti-HA (1:1,000) from Roche; rabbit anti-Egr-1 (1:200), rabbit anti-HSP70 (1:200), B-Raf (1:100), and anti-HSP90 (1:200) antibodies from Santa Cruz Biotechnology; and mouse anti-Bcl-2 (1:250) from BD Pharmingen.

Chromatin Immunoprecipitation Analysis—This was performed as described previously (16, 17). DNA primers used for detection of Bcl-2 P2 and Egr-1 promoters were described previously (16, 17).

RNA Preparation and Quantitative Real-time PCR—RNA was extracted using TRizol reagent (Invitrogen) according to the manufacturer’s directions. To make cDNAs, RNA (2 μg) was primed with oligo(dT) and reverse-transcribed with SuperScript reverse transcriptase (Invitrogen) according to the manufacturer’s directions. Quantitative real-time PCR was carried out using the GoTaq real-time PCR system (Promega) according to the manufacturer’s instructions. The primers used were as follows: ATF5, 5′-CCTCTACCCCTGCCACCC-3′ and 5′-CAGATGCTAGGTAGGGAGGA-3′; Bcl-2, 5′-CGACTTTGCGAGAGATGTCGA-3′ and 5′-ATGCCGTTTAGTACTCAG-3′; Egr-1, 5′-TGCACCCAACCTTTCTACT-3′ and 5′-AGGTTCCTCTTGTGTGGACG-3′; and β-actin, 5′-CATGC-TGGGCCGCCCTAGGC-3′ and 5′-GCCCCGTCTAGGCGC-3′. Cycling parameters are available upon request.

Luciferase Assay—A luciferase reporter assay was performed as described previously (17). Cell extracts were prepared 48 h after transfection or after serum deprivation or cpt treatment using lysis buffer provided in the luciferase system (Promega). Luciferase and Renilla activities were determined using a TD20/20 luminometer (Turner Designs). Relative luciferase activities were obtained by normalizing the luciferase activity against Renilla activity. Data are presented as mean ± S.E. (n = 3).

Clonogenic Assay—This was performed as described (28). Briefly, cells in 24-well plates were transfected with empty vectors or vectors expressing HSP70 and/or dnATF5. Twenty-four hours later, transfected cells were subjected to control treat-

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\(^3\) D. Liu, D. Qian, B. Wang, J. Yang, and Z. Lu, unpublished data.
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FIGURE 1. Regulation of ATF5 expression by HSP70 in C6 cells subject to various types of cellular stress and apoptotic stimulation. A and B, immunoblotting analysis monitoring expression of HSP70 and ATF5 in C6 cells treated with ARS (20 μM) (A) or Tg (2 μM) (B) for the indicated times. β-actin was used as a control in all immunoblotting analyses. The labels on the right of the blots indicate the positions of molecular markers. C, immunoblotting analysis monitoring expression of HSP70 and ATF5 in C6 cells treated with Tg at indicated concentrations for 8 h. D and E, immunoblotting analysis monitoring expression of HSP70 and ATF5 in C6 cells treated with ARS (20 μM) (D) or subject to serum deprivation (−Serum) (E) for indicated times. F and G, immunoblotting analysis monitoring expression of FLAG-ATF5 and HSP70 in C6 cells transfected with a vector expressing FLAG-ATF5 and an empty vector empty (−) or a vector expressing (+) HSP70 (F) or an empty vector (−) or a vector expressing (+) shRNA against HSP70 (G). H, immunoblotting analysis monitoring expression of endogenous ATF5 in C6 cells transfected with an empty vector (−) or a vector expressing HSP70 (+) following treatment with vehicle (−) or quercetin (+) (10 μM) for 8 h.

Concomitant Regulation of ATF5 and HSP70 in C6 and U87 Cells Subject to Apoptotic Stimulation—On the basis of reports that function of both ATF5 and HSP70 is required for survival of several types of cancer cells (8, 9, 14, 15, 30) and that both ATF5 and HSP70 are involved in cellular response to a variety of stress signals (6, 19, 20, 31–35), we hypothesized that the two stress-related molecules may act concertedly to support cell survival in glioma cells where both ATF5 and HSP70 are highly expressed (14, 36). As shown in Fig. 1, A and B, both ATF5 and HSP70 are transiently up-regulated in C6 cells exposed to ARS, an agent that elicits oxidative stress in a variety of cells, or Tg, a chemical that causes endoplasmic reticulum stress and is known to up-regulate HSP70. Induction of ATF5, and of HSP70, by Tg is also dose-dependent (Fig. 1C). Prolonged ARS treatment, however, reversed the up-regulation trend of both ATF5 and HSP70 in C6 cells (Fig. 1D and supplemental Fig. S1A). Similar transient up-regulation followed with down-regulation of ATF5 and HSP70 was also observed in C6 cells subject to serum deprivation or cpt treatment, two conditions that cause cell death (Fig. 1E and supplemental Fig. S1, B and C) and in human U87 glioma cells subject to Tg or ARS treatment (supplemental Fig. S1, D and E). These data indicate that ATF5 and HSP70 are concomitantly regulated in C6 and U87 glioma cells subject to a variety of apoptotic stimulations.

HSP70 Regulates ATF5 Expression in C6 and U87 Cells—To determine the role of HSP70 in ATF5 expression regulation, we analyzed the effect of HSP70 in the expression of a FLAG-tagged ATF5 using C6 cells cotransfected with a vector expressing FLAG-ATF5 and an empty vector or a vector expressing HSP70 or shRNA-HSP70. As shown in Fig. 1, F and G, overexpression of HSP70 dramatically increased FLAG-ATF5 expression, whereas knockdown of HSP70 led to depletion of the FLAG-ATF5. To examine the response of endogenous ATF5 to HSP70, we transiently transfected C6 or U87 cells with an empty vector (control) or a vector expressing HSP70 and treated the cells with or without quercetin, a drug that has been used to down-regulate endogenous HSP70 (37). Both HSP70 and ATF5 were similarly down-regulated by quercetin in C6 and U87 cells. In addition, overexpression of WT HSP70 blocked ATF5 down-regulation induced by quercetin in both cells (Figs. 1H and supplemental Fig. S1F). These data indicate that HSP70 regulates ATF5 expression and suggest that this regulation may play an important role in glioma cell survival promoted by HSP70.

Specific Interaction between ATF5 and HSP70—HSP70 is a chaperone protein that relies on ATP-regulated association to

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assist a large variety of protein folding processes. To determine whether ATF5 interacts with HSP70, we performed coimmunoprecipitation analyses using a C6 cell line stably expressing a FLAG-HA-tagged ATF5 (C6-FLAG-HA-ATF5). Because the ectopically expressed FLAG-HA-ATF5 is at near-physiological level compared with the endogenous ATF5 (17), interactions between this tagged FLAG-HA-ATF5 and other cellular proteins are likely to reflect those involving the endogenous ATF5. Immunoblotting of HA immunoprecipitates from the C6-FLAG-HA-ATF5 cells with an antibody against HSP70 showed that HSP70 was readily coimmunoprecipitated with FLAG-HA-ATF5 (Fig. 2A). Similarly, a reverse immunoprecipitation experiment showed that FLAG-HA-ATF5 is present in the HSP70 immunoprecipitates (Fig. 2A). Immunoblotting of HSP70 immunoprecipitate from C6 cells with an antibody against ATF5 further demonstrated that endogenous ATF5 and HSP70 interact with each other (Fig. 2B). A parallel coimmunoprecipitation analysis showed that HSP90, another HSP family member that functionally resembles HSP70, did not interact with ATF5, although it readily pulled down B-Raf (Fig. 2B), a known HSP90 substrate protein (38). These results indicate that ATF5 selectively interacts with HSP70 in C6 cells.

ATF5 has a N-terminal Pro-rich domain and a C-terminal basic zipper region. To determine which part of ATF5 interacts with HSP70, we performed an immunoprecipitation analysis using C6 cells cotransfected with a construct expressing HSP70-HA and a construct expressing GFP-ATF5 or GFP-dnATF5 that has the Pro-rich domain deleted and acts as dominant-negative (14, 26). Immunoblotting analysis of GFP immunoprecipitates with an HA antibody or immunoblotting of HA immunoprecipitates with a GFP antibody both showed that WT ATF5 but not dnATF5 interacted with HSP70 (Fig. 2C). These results indicated that HSP70 specifically interacts with the N-terminal Pro-rich domain of ATF5.

HSP70 Protects ATF5 from Rapid Protein Turnover Promoted by a Proteasome-dependent and Caspase-dependent Process—

One possibility for HSP70 to promote ATF5 expression is to increase the stability of the ATF5 protein, which is known to be subject to proteasome-dependent down-regulation (22). To test this possibility, we transfected C6 cells with an empty vector or a vector expressing shRNA-HSP70 and treated the transfected cells without or with MG132, an inhibitor of the 26 S proteasome. As shown in Fig. 3A and supplemental Fig. S2A, whereas depletion of HSP70 down-regulated ATF5 dramatically, and the presence of MG132 significantly reversed ATF5 down-regulation brought about by depletion of HSP70. Similar results were obtained using the C6-FLAG-HA-ATF5 cell line with lactacystin, another inhibitor of the 26 S proteasome (supplemental Fig. S2B). The partial reversal in ATF5 abundance in the presence of MG132 or lactacystin further suggested involvement of additional mechanisms. In consistency, addition of either Baf or zVADfmk, both of which are pan-caspase inhibitors, also partially inhibited the ATF5 down-regulation under the condition of HSP70 depletion (Fig. 3A and supplemental Fig. S2, A and B). Addition of both MG132 and Baf (Fig. 3A and supplemental Fig. S2A) or both lactacystin and zVADfmk (supplemental Fig. S2B) completely reversed the loss of ATF5 expression elicited by depletion of HSP70. These results indicated that ATF5 is subject to both proteasome-dependent and caspase-dependent protein degradation, and expression of HSP70 effectively inhibits both. To determine whether HSP70 additionally regulates ATF5 expression at mRNA level, we performed a quantitative real-time PCR analysis of the ATF5 mRNA in C6 cells transfected with an empty vector or a vector expressing shRNA-ATF5 or shRNA-HSP70 and treated without or with MG132 or Baf. As shown in Fig. 3B, whereas expression of shRNA-ATF5 down-regulated ATF5 mRNA as expected, expression of shRNA-HSP70, with or without the presence of MG132 or Baf, did not affect ATF5 mRNA level in C6 cells. These data thus ruled out the possibility that regulation of ATF5 expression by HSP70 involves transcription or mRNA regulation.

To further assess the effect of HSP70 on ATF5 protein stability in glioma cells, we determined the half-life of ATF5 in C6 and U87 cells with or without overexpression of HSP70. C6 and U87 cells were transiently cotransfected with pGFP-FLAG-ATF5 and an empty vector or a vector expressing HSP70-HA, and the decay of the ATF5 protein was determined in the presence of CHX, an inhibitor of protein translation. As shown in Fig. 3C, ATF5 was rapidly degraded in C6 cells transfected with the control vector, showing a half-life of 1 to 2 h. In contrast, ATF5 was markedly stabilized in C6 cells transfected with

![FIGURE 2. Interaction between ATF5 and HSP70. A, reciprocal immunoprecipitation (IP) analysis showing interaction between ATF5 and HSP70. The C6-pCIN4 and C6-pCIN4-FLAG-HA-ATF5 cell lines were used for cell extract preparation. The expression of endogenous HSP70 and FLAG-HA-ATF5 were detected using antibodies against HSP70 and HA as indicated (Input). Antibodies used for reciprocal IP and immunoblotting (IB) analyses for detection of associated proteins are as indicated. B, immunoprecipitation analysis showing that endogenous ATF5 interacts with HSP70 but not with HSP90. C6 cells were used for IP, and indicated antibodies were used in the immunoblotting analyses. B-Raf is a known HSP90 substrate protein and was used as a control. C, C6 cells transiently transfected with indicated constructs were used for IB and IP analyses as in A.](image-url)
HSP70-HA, increasing the half-life of ATF5 to more than 6 h. Similar results were observed using U87 cells (Fig. 3D).

Together, these results indicate that HSP70 interaction with ATF5 markedly increases ATF5 protein stability in glioma cells by protecting ATF5 from both proteasome-dependent and caspase-dependent protein degradation.

**HSP70 Requires ATPase Activity for its Interaction with and Stabilization of ATF5**—Previous studies showed that the association of HSP70 with substrate proteins requires a conformational rearrangement in HSP70 driven by an ATPase cycle (39, 40). To determine whether the interaction between HSP70 and ATF5 depends on the ATPase activity of HSP70, we performed an immunoprecipitation analysis using C6 cells cotransfected with pEGFP-FLAG-ATF5 and an empty vector (−) or a vector expressing (+) shRNA-HSP70 and treated with vehicle (−) or MG132 (10 μM) or BAF (50 μM) for 8 h. The amount of mRNA from cells transfected with empty vector (vector) was arbitrarily set at 100%. Data are presented as mean ± S.E. (n = 3).

**FIGURE 3.** HSP70 inhibits ATF5 degradation. A, immunoblotting analysis of ATF5 expression in C6 cells transiently transfected with an empty vector (−) or a vector expressing (+) shRNA-HSP70 and treated with vehicle (−) or MG132 (10 μM) or BAF (50 μM) for 8 h. Relative ATF5 density was determined against β-actin controls. The density of ATF5 in untreated cells was arbitrarily set at 1.0. B, quantitative real-time PCR analysis monitoring ATF5 mRNA abundance in C6 cells transfected with indicated constructs and treated with vehicle (−) or MG132 (10 μM) or BAF (50 μM) for 8 h. The amount of mRNA from cells transfected with empty vector (vector) was arbitrarily set at 100%. Data are presented as mean ± S.E. (n = 3). C, measurement of ATF5 turnover. C6 cells cotransfected with pEGFP-FLAG-ATF5 and an empty vector (−) or a vector expressing HSP70 (+) were incubated with or without CHX (10 μg/mL) for indicated times. Immunoblotting (IB) analysis and determination of relative ATF5 expression were carried out as in A except that anti-FLAG antibody was used for detection of FLAG-ATF5 and expression of GFP was used as control (upper panel). Western blot films from three independent experiments were scanned, and the relative density of protein bands was calculated using β-actin as a loading control (lower panel). Band density corresponding to CHX (0) was set at 100. Error bar depicts mean ± S.E. D, transfection and immunoblotting analysis were performed as in C except that U87 cells were used.

To determine whether HSP70-promoted ATF5 stabilization also depends on the ATPase of HSP70, we cotransfected C6 cells with pCIN4-FLAG-HA-ATF5 and an empty vector or a vector expressing HSP70 or HSP70(K71E) and analyzed ATF5 degradation in the presence of CHX. As shown in Fig. 4B, unlike expression of HSP70, which inhibited ATF5 down-regulation, expression of HSP70(K71E) had little effect. Similarly, expression of HSP70 but not HSP70(K71E) inhibited down-regulation of endogenous ATF5 in C6 cells subject to prolonged cellular
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FIGURE 5. Expression of HSP70 blocks down-regulation of ATF5 in C6 and U87 glioma cells subject to prolonged cellular stress. A and B, C6 cells transfected with empty vectors (−) or vectors expressing (+) HSP70 or HSP70(K71E) were subjected to serum deprivation for the indicated days (A) or cpt treatment for 2 days (B). Immunoblotting (IB) analysis was performed as in Fig. 1. C, immunoblotting analysis monitoring expression of HSP70 and ATF5 in C6 cells transfected with empty vectors (−) or vectors expressing HSP70 or HSP70(K71E) and subjected to ARS treatment for the indicated days. D, cell treatment and immunoblotting analyses were carried out as in C, except that U87 cells were used.

FIGURE 6. HSP70 modulates expression of Bcl-2 in C6 cells. A, C6 cells were transiently transfected with a luciferase reporter driven by the Bcl-2 P2 promoter, a Renilla vector, and an empty vector (−) or a vector expressing shRNA-HSP70 and an empty vector (−) or a vector expressing ATF5. The results are reported as relative light units and normalized with Renilla activity as an internal control. The relative luciferase activity of empty vector-transfected cells was arbitrarily set at 100. Data are presented as mean ± S.E. (n = 3). *, significance comparison between without and with overexpression of ATF5. p < 0.02. B, quantitative real-time PCR analysis monitoring Bcl-2 mRNA abundance in C6 cells transfected with the indicated constructs and/or subject to serum deprivation for 2 days. The amount of mRNA from cells transfected with empty vector (vector) and fed with serum was arbitrarily set at 100. Data are presented as means ± S.E. (n = 3). *, significance comparison between without and with overexpression of ATF5, p < 0.01. C and D, immunoblotting (IB) and ChIP assay monitoring expression of HSP70, ATF5, and Bcl-2 and binding of ATF5 to the Bcl-2 P2 promoter in C6 cells transfected with indicated constructs. In D, cell extracts and chromatin materials were prepared from transfected cells that were cultured in medium containing 10% serum (+) or deprived of serum (−) for 2 days.

stress by serum deprivation (Fig. 5A) or cpt treatment (B) and in C6 and U87 cells subject to ARS treatment (C and D), which were reproducibly observed in multiple experiments. These results indicate that both the interaction between HSP70 and ATF5 and the stabilization of ATF5 by HSP70 require active ATPase in the HSP70 molecule.

HSP70 Modulates Expression of ATF5-regulated Genes in C6 Cells—On the basis of our data that HSP70 directly regulates ATF5 abundance in C6 cells and U87 cells, we anticipated that HSP70 would also modulate in these cells the expression of Bcl-2 and Egr-1, two genes that we recently been found to be transactivated by ATF5 (16, 17). To examine whether HSP70 expression impacts Bcl-2 and Egr-1 expression at the transcription level, we performed a luciferase reporter assay using C6 cells cotransfected with a luciferase reporter driven by a Bcl-2 promoter or Egr-1 promoter, a Renilla vector, and an empty vector (−) or a vector expressing shRNA-HSP70 and an empty vector (−) or a vector expressing ATF5. The results support the conclusion that HSP70 stimulates ATF5-regulated genes in C6 cells.
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FIGURE 7. ATF5 plays an essential role in survival of C6 and U87 cells promoted by HSP70. A, C6 cells transfected with the indicated DNA constructs were subjected to serum deprivation for 2 days. After the cells were stained with Hoechst 33342, transfected (GFP +) cells were scored for the presence of apoptotic nuclei. Data are presented as mean ± S.E. (n = 3). *, significance comparison between without and with expression of dnATF5, p < 0.02. B, C6 cells transfected with empty vectors or vectors expressing HSP70 and/or dnATF5. 24 h later, transfected cells were subjected to mock treatment (+) or serum deprivation (−) for 3 days. 200 cells from each group were seeded in 60-mm culture dishes for clonogenic analysis. Only colonies with more than 20 cells were counted. Data are presented as mean ± S.E. (n = 3). *, significance comparison between without and with expression of dnATF5, p < 0.01. C, C6 cells transfected with the indicated DNA constructs were subjected to cpt treatment for 2 days. The apoptotic analysis was performed as in A. *, significance comparison between without and with expression of shRNA-ATF5, p < 0.02. D, cell treatment and transfection and apoptotic analysis were carried out as in A, except that U87 cells were used. *, significance comparison between without and with expression of dnATF5, p < 0.01. E, TUNEL assay of C6 cells transfected with the indicated DNA constructs and treated with or without quercetin (10 μM) for 24 h. Immunoblotting analysis monitoring activation of caspase-3 with an antibody specifically recognizing activated caspase-3 (top panel). β-actin was used as a loading control. Transfected (GFP +) cells were scored for the presence of TUNEL-positive cells (bottom panel). Data are presented as mean ± S.E. (n = 3). *, significance comparison between without and with overexpression of ATF5, p < 0.02.

DISCUSSION

In this study, we investigated the relationship of two important stress-related molecules, HSP70 and ATF5, in the rat C6 glioma and human U87 glioma cells. We showed that 1) HSP70 and ATF5 are concomitantly up- and down-regulated in these glioma cells, subject to a variety of cellular stress and apoptotic stimulation; 2) ATF5 is an extremely unstable protein whose turnover is facilitated by rapid protein degradation via both proteasome-dependent and caspase-dependent pathways; 3) HSP70 binds to the N-terminal Pro-rich domain of ATF5, and this binding blocks ATF5 protein degradation and dramatically stabilizes ATF5 in C6 and U87 glioma cells; 4) two previously identified ATF5 downstream target genes, Bcl-2 and Egr-1, are up-regulated by HSP70 in an ATF5-dependent manner; and 5) ATF5 mediates the prosurvival function of HSP70 in C6 and U87 glioma cells. Our findings reveal an essential role of HSP70 in posttranslational regulation of ATF5 and establish ATF5 as a major substrate protein of HSP70 that mediates the unique function of HSP70 in promoting cell survival of transformed cells.

In cancer cells, overexpression of HSP70 is thought to provide a survival advantage because it is able to interact with multiple components of both prosurvival and apoptotic pathways (41–45). Notably, HSP70 has the capacity to protect certain caspase substrates by directly binding to caspase-3 and caspase-9 (46, 47) or substrates themselves (48). It can also block ubiquitin-dependent protein degradation of certain pro-
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Survival molecules such as Bcl-2, Mcl-1, and nucleolin (32, 44, 49). Our study showed that ATF5 is an unusually unstable protein in C6 and U87 cells because of both proteasome-dependent degradation and caspase-dependent degradation (Fig. 3). Consistent with a complete dependent on HSP70 for ATF5 stability, we showed that HSP70 and ATF5 are concomitantly up- and down-regulated in C6 and U87 cells subject to a variety of apoptotic treatment (Fig. 1) and that HSP70 expression increases ATF5 expression by blocking both proteasome- and caspase-dependent processes (Fig. 3 and supplemental Fig. S2). Thus, HSP70 appears to be involved in multiple and redundant mechanisms to block the caspase- and proteasome-dependent degradation of ATF5.

The N-terminal region of ATF5 is both rigid and uniquely structured because of high proportion of prolines, and its C-terminal basic zipper region is only partially structured under physiological conditions (25). These features may necessitate the ATF5 molecule to partner with chaperone proteins for stability and could contribute to the extreme susceptibility of ATF5 to protein degradation mechanisms that provide protein quality control. It is likely that ATF5 is programmed for rapid destruction unless it is bound and protected by HSP70. In addition, given that HSPs are chaperones that catalyze the proper folding of both nascent proteins and the refolding of denatured proteins, HSP70 interaction with ATF5 and, therefore, ATF5 protection by HSP70 could be needed not only for the free, naturally “unstructured” ATF5 molecules but also for the nascent ATF5, whereas its N terminus begins to emerge from a translating ribosome. Furthermore, interaction of HSP70 with the N-terminal region of ATF5, which is the activation domain of the transcription factor, could impact the ability of ATF5 to interact with coactivators such as p300 (unpublished data) and, hence, its transactivation potential. Therefore, HSP70 may regulate the activity of ATF5 as well as its stability at multiple stages.

Although substrate binding is essential to accelerate the ATP hydrolysis activity of HSP70, the chemical energy of ATP is also required to perform mechanical work; the opening and closing of the substrate binding domain pocket on the HSP70 molecule and, therefore, binding of substrates (6, 39, 40). Consistent with a required role for ATPase in substrate binding, we found that, unlike the WT HSP70, the ATPase-dead HSP70(K71E) failed to interact with ATF5 and was unable to prevent ATF5 from rapid protein degradation (Figs. 4 and 5). These results suggest that ATF5 preservation is an ATP-driven process, and it could be significantly compromised in cells when ATP is depleted, as in the case of prolonged cellular stress.

Previous studies had shown that both HSP70 and ATF5 are required for survival of many types of transformed cells, whereas both are dispensable in non-transformed cells (8, 9, 14, 15). One reason for ATF5 to promote cancer-specific cell survival is its ability to differentially stimulate Bcl-2 expression in transformed cells but not at all in non-transformed cells (16). We showed in this study that HSP70 stimulates Bcl-2 expression through stabilization of ATF5 and that ATF5 is an obligatory mediator connecting HSP70 to Bcl-2 (Fig. 6). Because Bcl-2 is reportedly protected by HSP70 from ubiquitin-dependent degradation (44), our findings thus suggest that HSP70 promotes Bcl-2 up-regulation by multiple mechanisms in glioma cells.

In conclusion, our findings establish ATF5 as a substrate protein of HSP70 that contributes to the prosurvival function of HSP70 in C6 and U87 glioma cells. The HSP70-dependent ATF5 stabilization plays an essential role in the survival of C6 and U87 cells subject to various types of cellular stress and apoptotic stimulation. These data suggest exciting possibilities for the development of highly specific multimodal therapies for glioblastomas on the basis of inhibition of HSP70, ATF5, Egr-1, and Bcl-2 expression.

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