Hsp70 acetylation prevents caspase-dependent/independent apoptosis and autophagic cell death in cancer cells

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Abstract. Cancer cells are continuously challenged by adverse environmental factors including hypoxia, metabolite restriction, and immune reactions, and must adopt diverse strategies to survive. Heat shock protein (Hsp) 70 plays a central role in protection against stress-induced cell death by maintaining protein homeostasis and interfering with the process of programmed cell death. Recent findings have suggested that Hsp70 acetylation is a key regulatory modification required for its chaperone activity, but its relevance in the process of programmed cell death and the underlying mechanisms involved are not well understood. In this study, we sought to investigate mechanisms mediated by Hsp70 acetylation in relation to apoptotic and autophagic programmed cell death. Upon stress-induced apoptosis, Hsp70 acetylation inhibits apoptotic cell death, mediated by Hsp70 association with apoptotic protease-activating factor (Apaf)-1 and apoptosis-inducing factor (AIF), key modulators of caspase-dependent and -independent apoptotic pathways, respectively. Hsp70 acetylation also attenuated autophagic cell death associated with upregulation of autophagy-related genes and autophagosome induction. Collectively, these results suggest that the acetylation of Hsp70 plays key regulatory roles in cell death pathways as well as in its function as a chaperone, together enabling cellular protection in response to stress.

Introduction

Cancer cells are confronted with diverse environmental stresses including hypoxia, nutrient deprivation, and pH changes caused by metabolic byproducts and the tumour microenvironment (1-3). Stress factors induce diverse apoptotic signaling in cells, in which various pro-apoptotic proteins can be activated. To overcome the apoptotic response, cancer cells develop diverse ways to inhibit apoptotic signaling (2,4,5). Furthermore, these signaling alterations can also allow cancer cells to resist chemo- or radiotherapeutic challenge (6).

The heat shock protein (HSP) 70 chaperone system is upregulated in many cancers and facilitates the refolding or degradation of proteins that are denatured as a result of stress (7-9). In addition, Hsp70 can also directly interfere with apoptosis pathways to protect cells (10-12). The diverse protective mechanisms of Hsp70 are known to confer resistance to some forms of stress-induced cell death.

Several pro-apoptotic proteins that are directly inhibited by Hsp70 have been reported. Apoptotic protease-activating factor 1 (Apaf-1), a key regulatory component of the caspase-dependent apoptotic pathway, directly associates with Hsp70 (11,12). This interaction prevents oligomerization of Apaf-1 with procaspase-9, consequently inhibiting apoptosome formation. Another pro-apoptotic protein, apoptosis-inducing factor (AIF) is a mitochondrial intermembrane protein that initiates one of the key mechanisms of caspase-independent apoptosis (13). Under stress conditions, AIF, which is normally well secluded in mitochondria, translocates to the cytosol and ultimately to the nucleus where it induces caspase-independent peripheral chromatin condensation and DNA fragmentation (14).

Autophagic cell death is another type of programmed cell death, which involves autophagy (15,16). The crosstalk between autophagic and apoptotic cell death is a current topic of heightened interest. In relation to the HSP system, autophagy is a regulatory mechanism that maintains cellular protein homeostasis by sequestering and delivering large protein aggregates and whole damaged organelles to lysosomes for degradation (17). Previous studies have reported that autophagy is regulated by the heat shock response. Depletion of HSF-1 potentiates starvation-induced LC3 lipidation, which is associated with the formation of autophagosomal organelles, while Hsp70 overexpression inhibits this process (18).

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Recently, Hsp70 was reported to be acetylated by acetyltransferase arrest defective (ARD) 1 (Naa10, Nα-acetyltransferase 10) and this acetylation contributes to its protective function under cellular stress (19). Here, we sought to investigate the molecular mechanisms of Hsp70 acetylation-mediated cellular protection.

Materials and methods

Cell culture and stimulation. SH-SY5Y, HeLa and HEK293T cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO_2 humidified atmosphere at 37˚C. To induce cellular stress, cells were treated with 1 μg/ml doxorubicin or 0.3 mM H_2O_2 for 24 h.

Plasmid construction. Full-length cDNAs for human Hsp70 (Genbank: NM_005345.5) and human ARD1 (Genbank: NM_003491.3) were generated by PCR and subcloned into pCDNA3.1 (FLAG-ARD1) or pEGFP-C3 (GFP-Hsp70) vectors for cellular expression. For the construction of stable cell lines, cDNA constructs for Hsp70 and ARD1 were co-inserted into the pIRES vector, purchased from Clontech.

Transfection. Transfection was carried out as described previously (20). HEK293T cell transfection used polyethyleneimine (PEI) at a ratio of 4:1 (ml PEI/mg plasmid DAN) in basal media overnight, followed by a change of media. For the establishment of stable cells, pEGFP-C3-Hsp70 and pIRES-GFP-Hsp70-FLAG-ARD1 plasmids were transfected into SH-SY5Y cells using PolyFect reagent (Qiagen), according to the manufacturer's instructions. Transfected cells were maintained in complete DMEM with G418 (500 μg/ml). After several days, the surviving colonies were selected and amplified.

Antibodies. Anti-cleaved caspase-3 antibody (#9661, 1:3,000), anti-PARP antibody (#9542, 1:3,000), anti-Apaf-1 (#8969, 1:2,000) antibody, anti-AIF antibody (#4642, 1:2,000), anti-Atg12 antibody (#4180, 1:3,000), anti-Beclin-1 antibody (#3495, 1:3,000), and anti-LC3A/B antibody (#12741, 1:200) were purchased from Cell Signaling Technology. Anti-Myc antibody (9E10, sc-40, 1:3,000) and anti-lamin A antibody (4A58, sc-71481, 1:2,000) were purchased from Santa Cruz. The anti-FLAG antibody (M2, F1804, 1:3,000), anti-tubulin antibody (DM1A, T9016, 1:3,000) and anti-β-actin (A2066, 1:3,000) antibody were purchased from Sigma. The anti-GFP antibody (ab6556, 1:3,000) was from Abcam.

Nuclear fractionation. Cultured cells were washed with PBS, homogenized in buffer A [10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40], and centrifuged at 600 x g for 10 min at 4˚C. The nuclear pellet was washed with buffer A, resuspended in buffer C [10 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF, 0.2 mM EDTA, and 420 mM NaCl], and centrifuged for 30 min at 15,000 rpm, before the supernatant was isolated.

Immunoblotting and immunoprecipitation. Proteins were extracted using lysis buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100 and a protease inhibitor cocktail (Roche). Then, 20 mg of cell extract was used for immunoblotting. For immunoprecipitation, 1 mg of protein was incubated with a corresponding antibody conjugated to A or G beads (Upstate) overnight at 4˚C. Beads were washed three times with washing buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl and 0.1 mM EDTA. Following SDS-PAGE, membranes were immunoblotted using the corresponding primary antibody overnight at 4˚C. HRP-conjugated secondary antibodies were incubated with the membranes for 1 h at room temperature. Visualization was performed using ECL Plus (Intron) and LAS-4000 (GE Healthcare).

Immunocytochemistry. HeLa cells were seeded onto glass coverslips in 24-well plates and transfected with GFP-Hsp70 WT and K77R. After treatment of 0.3 mM H_2O_2 for 24 h, cells were fixed in 4% PFA for 20 min and permeabilized in 0.3% Triton X-100 in PBS for 5 min at room temperature. Then, cells were incubated with LC3A/B antibody (Cell Signaling Technology, #12741, 1:200) and visualized with Alexa 546-conjugated IgG (Molecular Probes). Nuclear staining was performed using Hoechst 33342 (Molecular Probes). The immunofluorescence was visualized using an Axiovert M200 microscope (Carl Zeiss).

Cell viability assay. Cell viability was calculated by measuring the amount of lactate dehydrogenase (LDH) released from the cells into the medium. Conditioned media from cultured cells were collected, and LDH activity was determined with an LDH assay kit (DoGen). Total cellular LDH activity was measured by solubilizing the cells with 0.2% Triton X-100.

Statistical analysis. Results are expressed as the means ± SD P-values were calculated by applying the two-tailed Student's t-test. A difference was considered statistically significant at $P<0.05$.

Results

Hsp70 acetylation protects cancer cells from doxorubicin-induced cell death. Hsp70 acetylation at residue K77 has been previously reported to protect cells from stress. To confirm the protective effect of Hsp70 acetylation, we treated Hsp70 wild-type (WT) and K77R mutant-expressing SH-SY5Y cells with doxorubicin and analyzed cell viability. Consistent with a previous report, overexpression of wild-type Hsp70 protected cells against doxorubicin-induced cell death, but this protective effect was eliminated by the presence of the K77R mutation (Fig 1A). The acetylation of Hsp70 at K77 is mediated by acetyltransferase ARD1 (19). To further validate the relevance of ARD1 in the protective function of Hsp70, ARD1 WT and a dominant-negative mutant (DN) that does not harbor acetyltransferase activity was co-expressed with Hsp70 constructs in SH-SY5Y cells, and cell viability was assessed after doxorubicin treatment (21). As expected, co-expression of the dominant-negative mutant ARD1 abolished the protective effect of Hsp70 WT (Fig. 1B), indicating that ARD1-mediated Hsp70 acetylation protects cancer cells from doxorubicin-induced cell death.

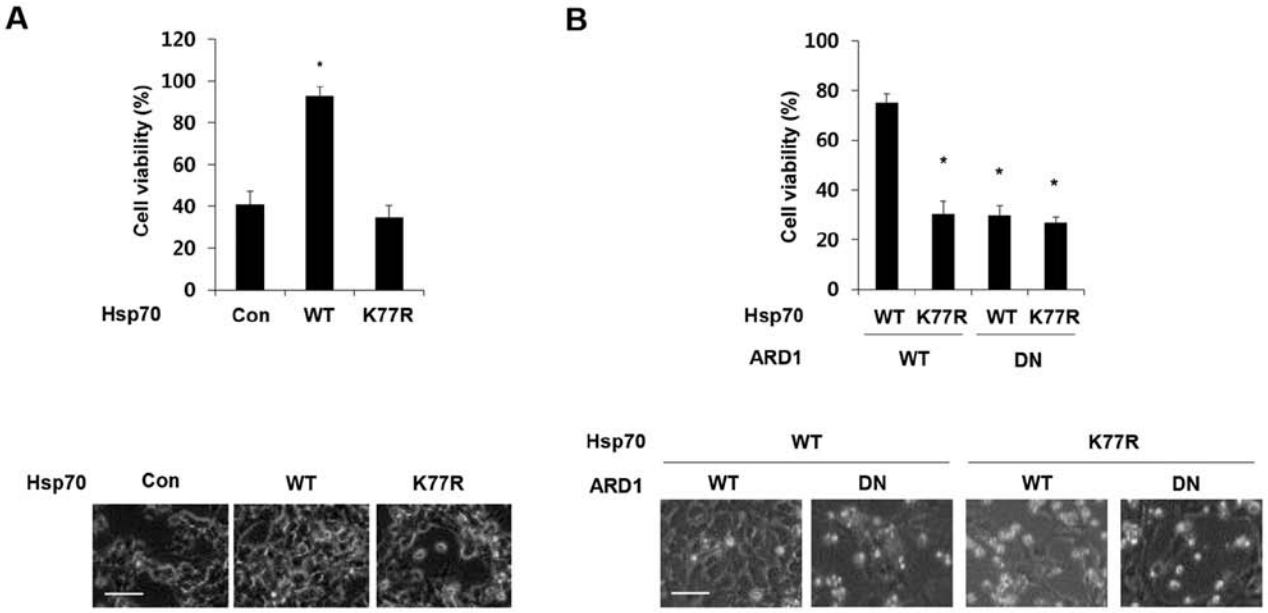

Figure 1. Hsp70 acetylation inhibits doxorubicin-induced cancer cell death. (A) Hsp70 acetylation promotes cell survival during doxorubicin challenge. SH-SY5Y cells expressing GFP-Hsp70 WT and K77R were treated with 1 μg/ml doxorubicin for 24 h and cell survival was assessed. Error bars indicate SD (n=3). *P<0.05 versus control (t-test). Scale bar, 50 μm. (B) ARD1 promotes cell survival via Hsp70 acetylation following doxorubicin treatment. FLAG-ARD1 WT and dominant negative (DN) forms were co-expressed with GFP-Hsp70 in SH-SY5Y cells. Cell viability was measured after 24-h treatment of 1 μg/ml doxorubicin. Error bars indicate SD (n=3). *P<0.05 versus Hsp70 WT/ARD1 WT (t-test). Scale bar, 50 μm.

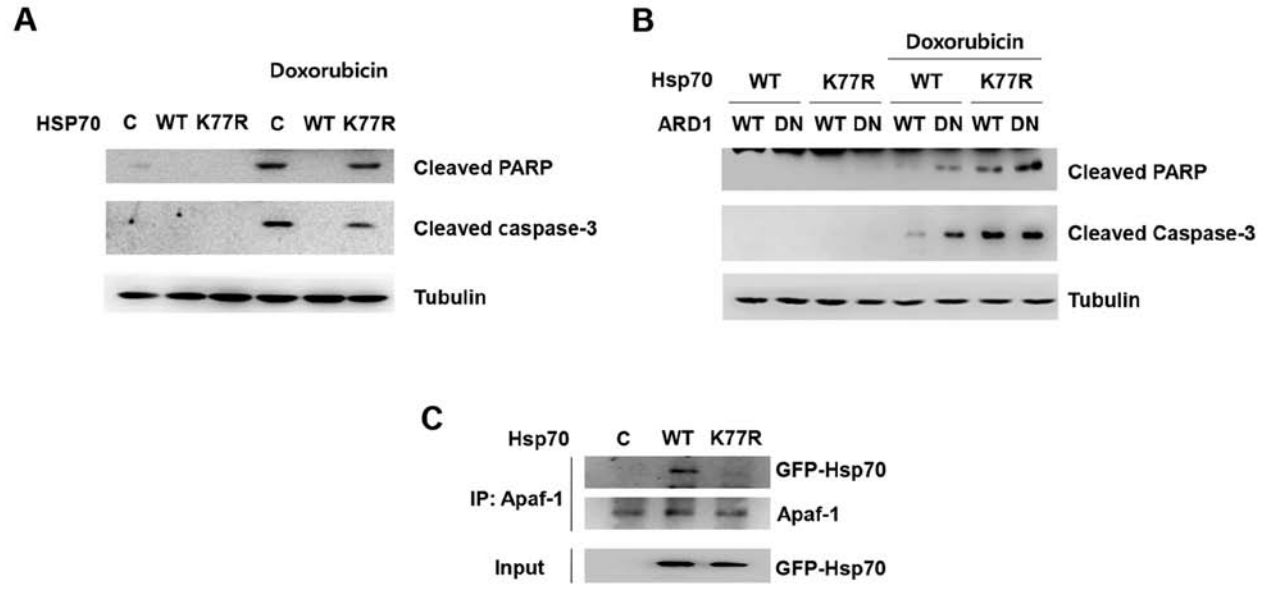

Figure 2. Hsp70 acetylation prevents caspase-dependent apoptotic cell death. (A) Hsp70 acetylation inhibits stress-induced cleavage of caspase-3 and PARP. After treatment of 1 μg/ml doxorubicin for 24 h, caspase-3 and PARP cleavage was assessed by western blotting. (B) ARD1 inhibits caspase-3 and PARP cleavage via Hsp70 acetylation. SH-SY5Y cells co-expressing FLAG-ARD1 and Hsp70 were treated with 1 μg/ml of doxorubicin for 24 h, and caspase-3 and PARP cleavage was analyzed. (C) Hsp70 acetylation is required for Apaf-1 binding. Apaf-1 was precipitated from HEK293T cells overexpressing GFP-Hsp70 WT and K77R, and the co-precipitated GFP-Hsp70 was quantified.

Hsp70 acetylation inhibits caspase-dependent apoptosis. To further investigate the underlying mechanisms of Hsp70 acetylation-mediated cellular protection, we first analyzed caspase-dependent apoptosis. Doxorubicin treatment induced cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), which are hallmarks of apoptosis (Fig. 2A). Consistent with the doxorubicin-induced cell death shown in Fig. 1, Hsp70 WT overexpression prevented doxorubicin-induced cleavage of caspase-3 and PARP, whereas the presence of the K77R mutation abolished the protective effect. Moreover, co-expression of the dominant-negative mutant ARD1 diminished Hsp70 acetylation-mediated inhibition of PARP and caspase cleavage (Fig. 2B).

Since Hsp70 is reported to inhibit apoptosome formation via binding with Apaf-1, a key molecule of caspase-dependent apoptosis, we next investigated whether acetylation at K77 can

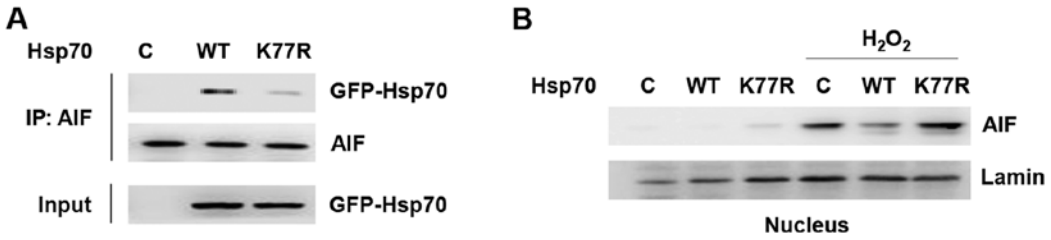

Figure 3. Hsp70 acetylation inhibits AIF-dependent apoptosis. (A) Hsp70 acetylation is required for AIF binding. AIF was precipitated from HEK293T cells, and co-precipitation of GFP-Hsp70 WT or K77R was assessed by western blotting. (B) Hsp70 acetylation inhibits nuclear translocation of AIF. SH-SY5Y cells expressing GFP-Hsp70 WT or K77R were treated in the presence or absence of 0.3 mM H_2O_2, and nuclear proteins were fractionated and assessed by western blotting.

modulate Apaf-1 binding. Hsp70 WT or the K77R mutant was overexpressed in HEK293T cells and their affinity to Apaf-1 was assessed by co-immunoprecipitation. Consistent with previous reports, Hsp70 wild-type was co-immunoprecipitated with Apaf-1 in cell extracts. Interestingly, however, mutation at K77 abrogated its binding to Apaf-1, implying that Hsp70 acetylation contributes to Hsp70/Apaf-1 association, subsequently leading to inhibition of functional apoptosome assembly and caspase activation (Fig. 2C). These results suggest that Hsp70 acetylation enables the association of Apaf-1 with Hsp70 and prevents apoptosis.

Hsp70 acetylation prevents caspase-independent apoptosis. Another cell death pathway in which Hsp70 is involved is AIF-dependent apoptosis. Hsp70 inhibits caspase-independent cell death by sequestering AIF and blocking its induction of apoptosis. To examine its relevance to caspase-independent apoptosis, we analyzed AIF binding to Hsp70 WT and the K77R mutant by co-immunoprecipitation (Fig. 3A). Interestingly, compared to Hsp70 WT, AIF binding to the K77R mutant was significantly reduced, implying that Hsp70 acetylation can interfere with AIF-mediated caspase-independent apoptosis.

We next verified whether this change in binding affinity was indeed relevant for AIF function. Nuclear translocation of AIF is a key final step in the AIF pathway (14). To validate the difference in binding affinity and whether it influences AIF nuclear translocation, Hsp70-expressing cells were treated with hydrogen peroxide and nuclear translocation of AIF was greatly increased (Fig. 3B). Moreover, Hsp70 WT reduced H_2O_2-induced nuclear translocation of AIF but the K77R mutant could not. These results demonstrate that Hsp70 K77 acetylation suppresses apoptosis by preventing AIF from nuclear translocation, consequently inhibiting caspase-independent cell death. Collectively, these findings indicate that Hsp70 acetylation protects cancer cells against apoptosis through inhibition of both caspase-dependent and -independent apoptotic pathways.

Hsp70 acetylation attenuates autophagic cell death. Autophagic cell death is another type of programmed cell death that is controlled by Hsp70. Recent studies of stress-induced Hsp70 acetylation and its modulatory role in autophagosome formation led us to hypothesize that Hsp70 acetylation at K77 can also regulate autophagy (22). To test this, we treated H_2O_2 to Hsp70 WT and K77R mutant-expressing cells and assessed autophagy-related proteins. Autophagy-related genes (Atg) are universal markers for autophagic induction (23). During stepwise autophagy induction, Beclin-1 (the mammalian orthologue of yeast Atg6)-mediated core complex formation and Atg12-Atg5 conjugation are key processes, in nucleation and elongation of autophagosome formation, respectively (24,25). Consistent with previous reports, Hsp70 WT overexpression decreased H_2O_2-induced Beclin-1 and Atg12-Atg5 conjugation, suggesting that Hsp70 plays a regulatory role in the modulation of autophagy (Fig. 4A). However, the Hsp70 K77R mutant could not prevent autophagy as demonstrated by an increase in Beclin-1 and Atg12-Atg5 conjugation. This observation implies that Hsp70 acetylation contributes to the attenuation of autophagic cell death.

We also analyzed microtubule-associated protein light chain 3 (LC3) to monitor autophagic induction. Upon autophagy, the unconjugated cytosolic form of LC3-I is converted to the phosphatidylethanolamine-conjugated form of LC3-II that forms the autophagosomal membrane (26,27). Therefore, the transition of LC3 from a diffusive cytoplasm pattern to the punctated membrane pattern is a hallmark of autophagy induction, indicating the formation of autophagic vacuoles (27). When compared to WT, the Hsp70 mutant caused an increase in LC3 expression and perinuclear autophagic vacuole formation (Fig. 4B). These results suggest that Hsp70 acetylation may play a role in the prevention of autophagic cell death.

Discussion

In response to stress, Hsp70 acetylation on K77 residue facilitated Hsp70 interaction with Apaf-1 and AIF, and inhibited Apaf-1 and AIF-dependent apoptosis. Moreover, acetylation of Hsp70 attenuated autophagy, observed via Atg12-Atg5 complex formation, Beclin-1 expression and perinuclear LC3 puncta formation, resulting in the inhibition of autophagic cell death. Taken together, our results suggest that Hsp70 acetylation inhibits cell death by at least three different mechanisms: i) attenuation of caspase-dependent pathways by interacting with Apaf-1 and blocking apoptosome formation, ii) inhibition of caspase-independent pathways by interacting with AIF and preventing its nuclear translocation, and iii) attenuation of autophagic cell death (Fig. 5).

Previously, Hsp70 acetylation induced by cellular stress was reported to increase its protein refolding chaperone activity. This is mediated by increased association of Hsp70 with co-chaperones assisting protein refolding such as Hsp90

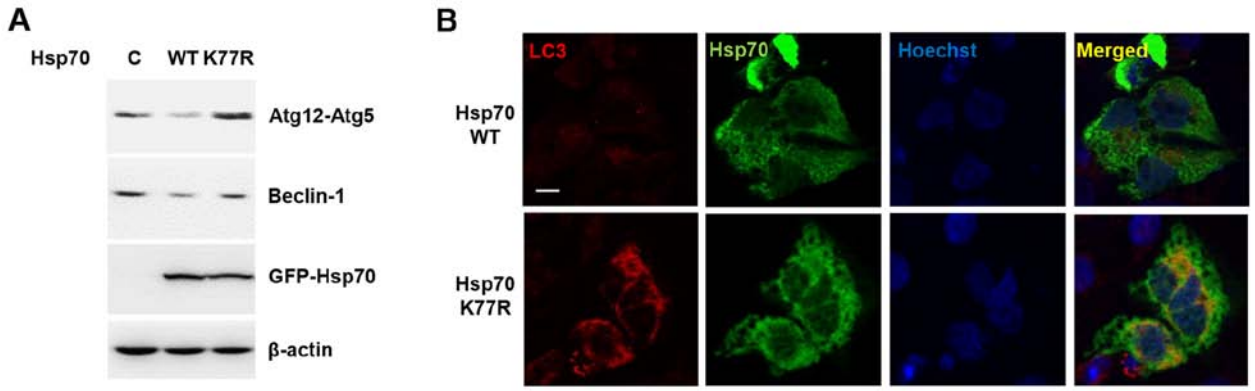

Figure 4. Hsp70 acetylation attenuates autophagic cell death. (A) Hsp70 acetylation reduces autophagy induction. Hsp70 WT and K77R-expressing SH-SY5Y cells were treated with 0.3 mM H_2O_2 for 24 h, and cell lysates were subjected to western blotting. (B) Hsp70 acetylation attenuates stress-induced autophagosome formation. Hsp70 WT and K77R-expressing HeLa cells were treated with 0.3 mM H_2O_2 for 24 h, and LC3 patterns were assessed by immunofluorescence staining. Nuclei were labeled with Hoechst (blue). Scale bar, 10 μm.

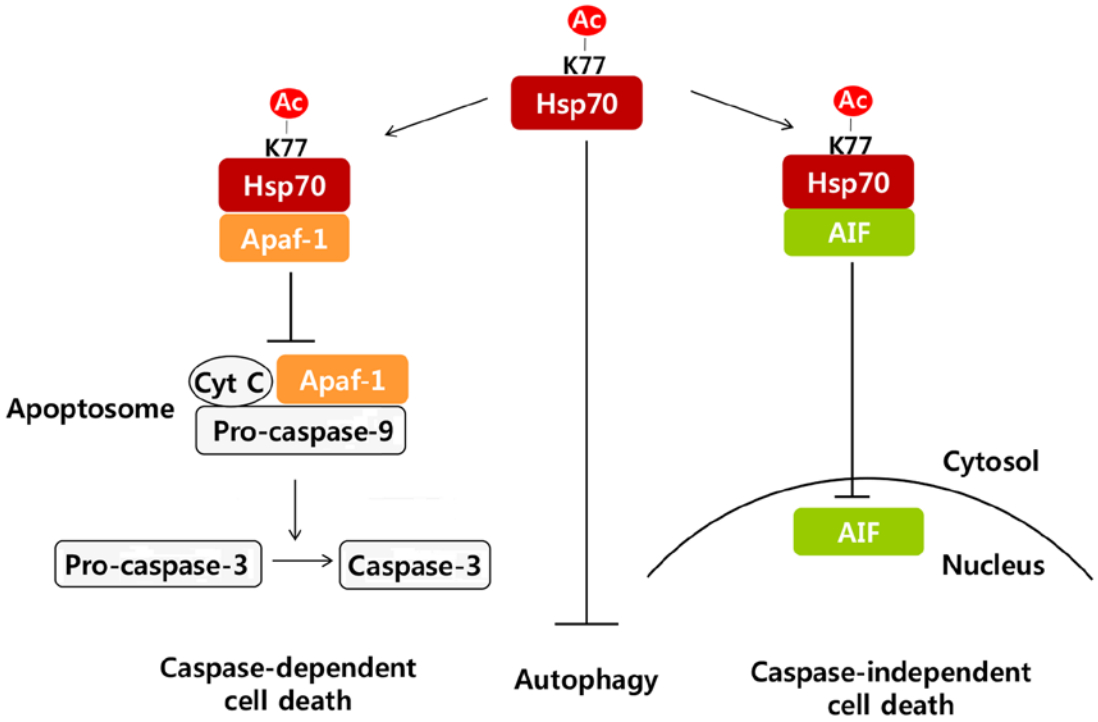

Figure 5. Model of prevention of cell death regulated by Hsp70 acetylation at K77. During stress, Hsp70 acetylation inhibits cell death by facilitating Apaf-1 and AIF binding to prevent caspase-dependent and -independent apoptosis and attenuating autophagic cell death.

and Hop (19). In addition to co-chaperone binding, in this study, Hsp70 acetylation facilitated binding of pro-apoptotic proteins as well, implying a broad impact for Hsp70 acetylation on its overall functionality.

Autophagy, together with HSP systems, represents a major protein quality control system. To cope with stress-induced cell damage, Hsp70 maintains protein homeostasis primarily by facilitating protein refolding and prevent aggregation, while autophagy results in protein and whole-organelle degradation. However, the role of autophagy in cell death and survival has long been controversial (3,28). It has been accepted as a cell survival mechanism in response to cellular stresses like starvation. However, recent molecular approaches have provided evidence that autophagy contributes to programmed cell death (29,30). Hsp70 has been suggested to play a crucial role in autophagy regulation, although the underlying mechanisms need further investigation (18,22). This study elucidates Hsp70 acetylation as a new regulatory mechanism in autophagic induction and also adds evidence for the contribution of autophagy to programmed cell death. Furthermore, it also suggests the possible linkage between HSP and the autophagy system mediated by Hsp70 acetylation, although the precise causality in physiological signals and underlying mechanisms requires further investigation.

How Hsp70 acetylation at K77 can affect its target protein affinity is another issue that needs to be addressed. The nucleotide binding domain (NBD) of Hsp70 that contains K77 is required for Hsp70/Apaf-1 interaction, whereas the

Hsp70/AIF interaction appears to be independent of NBD (13). Previously, we suggested that K77 acetylation in NBD may induce allosteric conformational changes in other domains of Hsp70, resulting in overall changes to target protein binding. The significant location of acetylation site K77 at interdomain contacts increases the interesting possibility that acetylation may modulate the Hsp70 conformational changes important for its protein domain interactions and overall activity. Although detailed studies are needed to elucidate the exact mechanisms involved, our results provide insight into the acetylation-mediated allosteric regulation of Hsp70.

In conclusion, we have described cancer cell survival mechanisms mediated by Hsp70 acetylation under stress. The findings offer rationale for the development of an Hsp70 inhibitor that minimizes disturbance of the normal cellular function of Hsp70. Regulation of Hsp70 K77 acetylation might be helpful in treating various diseases that involve Hsp70, including cancer, inflammatory diseases and neurodegenerative diseases.

Acknowledgements

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