BAG-1 Proteins Protect Cardiac Myocytes from Simulated Ischemia/Reperfusion-induced Apoptosis via an Alternate Mechanism of Cell Survival Independent of the Proteasome*

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BAG-1 (Bcl-2-associated athanogene-1) proteins interact with the HSC70 and HSP70 heat shock proteins and have been proposed to promote cell survival by coordinating the function of these chaperones with the proteasome to facilitate protein degradation. Consistent with this proposal, previous analyses in cancer cells have demonstrated that BAG-1 requires protein domains important for HSC70/HSP70 and proteasome binding in order to interfere with the growth inhibition induced by heat shock (Townsend, P. A., Cutress, R. I., Sharp, A., Brimmell, M., and Packham, G. (2003) Cancer Res., 63, 4150–4157). Moreover, cellular stress triggered the relocation of the cytoplasmic BAG-1S (~36 kDa) isoform to the nucleus, and both BAG-1S and the constitutively nuclear localized BAG-1L (~50 kDa) isoform suppressed heat shock-induced apoptosis to the same extent, suggesting a critical role in the nucleus. Because ischemia (I) and reperfusion (R) are important stress signals in acute and chronic heart disease, we have examined the expression and function of BAG-1 proteins in primary cardiac myocytes (CMs) and the Langendorff-perfused intact heart. The expression of both BAG-1 isoforms, BAG-1S and BAG-1L, was rapidly induced following ischemia in rat CM, and this was maintained during subsequent reperfusion. In control hearts, BAG-1S and BAG-1L were readily detectable in both the nucleus and the cytoplasm. However, BAG-1S did not relocate to the nucleus following simulated I/R. BAG-1 interacted with both RAF-1 and HSC70 in CMs and the whole heart, and binding to HSC70 was increased following I/R. Overexpression of the human BAG-1S and BAG-1 M isoforms significantly reduced CM apoptosis following simulated I/R. By contrast, BAG-1L or BAG-1S fused to a heterologous nuclear localization sequence failed to protect CM. Finally, overexpression of BAG-1 deletion and point mutants unable to bind HSC70/HSP70 failed to offer cardioprotection. Surprisingly, a deletion mutant lacking the N-terminal ubiquitin-like domain, which mediates interaction with the proteasome, still promoted cardioprotection. Therefore, BAG-1 has a novel cardioprotective role, mediated via association with HSC70/HSP70, which is critical upon cytoplasmic localization but independent of the BAG-1 ubiquitin-like domain. Our studies demonstrate that BAG-1 can influence cellular response to stress by multiple mechanisms, potentially influenced by the cell type and nature of the stress signal.

BAG-1 is a multifunctional protein that regulates important cellular control pathways and protects cells from a range of apoptotic stimuli (for recent reviews, see Refs. 1–3). Cells express multiple BAG-1 isoforms through alternate translation initiation of a single mRNA. In human cells, BAG-1S (~36 kDa) is preferentially localized in the cytoplasm, whereas BAG-1L, the largest isoform (~50 kDa), contains an N-terminal nuclear localization sequence (NLS) and is generally localized in the nucleus. A 46-kDa isoform, BAG-1M, partitions itself between the nucleus and the cytoplasm but is not expressed in some other species. BAG-1 isoforms are generated by alternate translation of a single mRNA, and BAG-1S translation is at least partially mediated via an internal ribosome entry segment (4). BAG-1 isoforms contain various domains, including repeats rich in acidic amino acid residues, an ubiquitin-like domain (ULD), and a C-terminal evolutionarily conserved “BAG” domain. The ULD is required for interaction with the proteasome, the major non-lysozymal proteolytic complex in cells (5, 6), whereas the BAG domain mediates interaction with the HSC70 and HSP70 molecular chaperones (7, 8) and the RAF-1 kinase (9, 10). BAG-1 modulates the chaperone activity of HSC70/HSP70, and BAG-1 has been proposed to link these heat shock proteins to the proteasome to facilitate protein degradation (5). Consistent with this idea, chaperone function is required for some ubiquitination reactions (5, 11, 12), and BAG-1 also interacts with Siah and CHIP, E3 ubiquitin ligases that catalyze the final step in the covalent attachment of ubiquitin moieties to specific substrate proteins, targeting them for proteasome-dependent degradation (6). The mechanism(s) by which BAG-1 promotes cell survival have been studied most intensively in cancer cell lines. Con-
Isoform-specific, BAG-1-mediated Cardioprotection

consistent with the model in which BAG-1 may facilitate protein turnover, we have recently demonstrated that suppression of stress-induced growth inhibition by BAG-1 in breast cancer cells requires specific amino acid residues within the BAG domain required for interaction with HSC70/HSP70 and the ULD (13). Although they were differentially localized in cells, all BAG-1 isoforms were equally effective in suppressing heat shock-induced growth inhibition. However, cellular stress promoted the nuclear accumulation of BAG-1 isoforms and chaperones, indicating that protection from apoptosis in these systems is dependent on a nuclear function of BAG-1. It is important to note that function and expression of chaperones and activity of the proteasome are frequently altered in malignant cells (14). Thus, these interactions might be particularly relevant in the setting of malignant disease; however, the function of BAG-1 has not been studied in non-transformed cells.

There are alternate mechanisms by which BAG-1 might promote cell survival. For example, the BAG domain of BAG-1 also mediates interaction with the RAF-1 kinase, and BAG-1 stimulates RAF-1 kinase activity independent of RAS. The binding of RAF-1 and chaperones is competitive, and in hamster fibroblasts elevated levels of HSP70 associated with cellular stress sequester BAG-1, leading to a decrease in RAF-1 activity. Thus, BAG-1 may function to coordinate cell growth signals following cellular stress (9). However, the significance of RAF-1 for BAG-1-mediated survival remains unclear. It is possible that BAG-1 may promote cell survival by stimulating RAF-1 dependent anti-apoptotic signaling pathways. Alternately, RAF-1 is also important for cell proliferation, and elevated levels of chaperones might therefore protect cells from apoptosis by allowing cells to accumulate in a less proliferative, apoptosis-prone state that might therefore protect cells from apoptosis by allowing cells to accumulate in a less proliferative, apoptosis-prone state by interfering with the activation of RAF-1 via BAG-1. Chronic and acute heart diseases are major health problems in the Western world, accounting for one-fifth of the total deaths worldwide, and are responsible for nearly one million deaths per year in the United States and 240,000 in the United Kingdom (15, 16). Apoptosis has become increasingly recognized as one of the mechanisms of cell death during injury following ischemia/reperfusion (I/R) in the heart (17). In human studies, apoptosis has been observed in the infarcted region of the heart following acute myocardial infarction (18), and activated caspases have been identified in the intact heart and from cultured cardiac myocytes following I/R injury (19). Although extensive necrosis is frequently observed in diseased heart, it is thought to arise subsequent to apoptosis, i.e. secondary necrosis. Existing therapies provide some protection for the ischemic myocardium against cell loss and functional impairment, but this protection is partial. The development of more effective strategies to maintain myocardial cell survival in ischemic heart disease remains a major goal. A detailed knowledge of the role of the pro- and anti-apoptotic proteins that modulate cardiac myocyte cell death will provide further understanding of the pathology of severe heart failure at the molecular level and provide new insights to novel anti-apoptotic therapies.

Here we demonstrate that BAG-1 has a novel cytoprotective role in the heart, decreasing apoptosis in primary cardiac myocytes following simulated I/R. In contrast to previous studies, in breast cancer cells only BAG-1S suppressed apoptosis, and this was dependent on its cytoplasmic localization. Interestingly, as in other cells, BAG-1 function required amino acid residues important for interaction with HSC70/HSP70, but, unlike other systems, BAG-1-mediated cardioprotection does not require the ULD, suggesting a novel mechanism of action that is independent of the proteasome.

EXPERIMENTAL PROCEDURES

1/R of Primary Neonatal Cardiac Myocytes—Ventricular cardiac myocytes (CMs) were isolated from neonatal Sprague-Dawley rats <2 days old and cultured as described previously (20, 21). Cultures consisted of >95% myocytes as determined by indirect staining with a monoclonal mouse antibody to desmin. For simulation of ischemia, the medium of cells was replaced with modified Esumi ischemic buffer (137 mM NaCl, 12 mM KCl, 0.49 mM MgCl2, 0.9 mM CaCl2, 0.4 mM HEPES, and 10 mM glucose, pH 7.4) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C for 4 h. Untreated cells were cultured in Esumi control buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl2, 0.9 mM CaCl2, 0.4 mM HEPES, and 10 mM glucose, pH 7.4) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C for 4 h. After completion of the hypoxic treatment, the cells were washed with 1× phosphate-buffered saline, normal medium was added, and the cells were returned to a standard incubator for a further 4 or 24 h of reoxygenation to simulate reperfusion (23).

Animal Model—Hearts from anesthetized adult male Sprague-Dawley rats were mounted in a Langendorff perfusion apparatus and simulated I/R performed as described previously (20). Isolated hearts were divided into two groups. Treated hearts were exposed to 30 min of regional ischemia achieved by occlusion of the left coronary artery, and subsequently reperfused for 2 h. Control hearts were continuously perfused for 2 h.

Western Blotting and Immunoprecipitations—For Western blotting, primary CMs were lysed in 1× radioimmune precipitation assay (RIPA) buffer (50 mmol/liter Tris- HCl, pH 8, 150 mmol/liter NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and 1% (v/v) Nonidet P-40) containing protease inhibitors. For immunoprecipitations, cells or tissue were lysed following standard protocols, and BAG-1 was precipitated overnight at 4 °C (24). For the experiments with HEK293 cells, which were used to model the BAG-1 domain helix mutants and their effects upon binding to chaperones, the cells were grown in Dulbecco’s modified Eagle’s media containing 10% fetal calf serum and antibiotics. When required, 5 × 106 cells were transfected for 24 h with 12.5 μg of DNA using the FuGENE 6 reagent (Roche Applied Science) at a ratio (v/v) of 3:1. Western blotting was performed following standard protocols with equal amounts of protein loaded for each sample (23, 25, 26). The antibodies used were polyclonal anti-BAG-1, m10, or TB2 (25) and mouse monoclonal anti-HSP70 (clone C92F3A-5) from StressGen; mouse monoclonal anti-HSCT (clone B6), rabbit polyclonal anti-actin (I-19), and mouse monoclonal anti-fibrinogen (clone 5H8) from Chemicon.

Cell Fractionation—Nuclear and cytosolic fractions were obtained by hypotonic lysis and differential centrifugation (25, 26). A lactate dehydrogenase (LDH) assay (Sigma) was used to monitor cytosolic contamination of the nuclear fraction. Western blotting for PCNA and GAPDH were further used to verify the purity of nuclear and cytoplasmic fractions, respectively.

Expression Constructs—Optimized Human BAG-1, -1M, and -1L expression constructs and expression constructs for the BAG-1S deletion mutants BAG-1S-(1-155) and BAG-1S-(89-230), the BAG-1S point mutant BAG-1S-(K50A), and BAG-1S fused to an heterologous NLS (NLS-BAG-1S) were generated by PCR and are described elsewhere (13, 27). The mutant codons underlined: H1, 5'-GAAACGTCGACGAGAAAGCGGTTGAGACTA-GCGGAGTTTGAACACCTTTG3'- (top) and 5'-CCAAATGGTTTAGACCACTTTGA-GCGAGTTTGAACACCTTTG3'- (bottom) for the H1 mutant; H1, 5'-GAACTAGTGTTTGGAGTACCAGAGGTAAAGGAGCTTGTGAGAGAGTGAGTGCAG-3' (top) and 5'-CTCTCTCTAGACGACCGATGCTCAGAAACGACCTTTG3'- (top) and 5'-CTCTCTCTAGACGACCGATGCTCAGAAACGACCTTTG3'- (bottom) for the H1 mutant (230), the BAG-1S point mutant BAG-1S-(E112A/K121A), the BAG-1S point mutant BAG-1S-(E112A/R121A), the BAG-1S point mutant BAG-1S-(G116A), the BAG-1S point mutant BAG-1S-(Q169A/K172A) (helix 2; H2), and the BAG-1S point mutant BAG-1S-(Q201A/D208A) (helix 3; H3) were generated using the QuikChange protocol (Stratagene) and the primers described as follows, with the mutated codons underlined: H1, 5'-GAAACGTCGACGAGAAAGCGGTTGAGACTA-GCGGAGTTTGAACACCTTTG3'- (top) and 5'-CCAAATGGTTTAGACCACTTTGA-GCGAGTTTGAACACCTTTG3'- (bottom) for the H1 mutant and the BAG-1S point mutant BAG-1S-(K50A), and BAG-1S fused to a heterologous NLS (NLS-BAG-1S) were generated by PCR and are described elsewhere (13, 27). The mutant codons underlined: H1, 5'-GAAACGTCGACGAGAAAGCGGTTGAGACTA-GCGGAGTTTGAACACCTTTG3'- (top) and 5'-CCAAATGGTTTAGACCACTTTGA-GCGAGTTTGAACACCTTTG3'- (bottom) for the H1 mutant and the BAG-1S point mutant BAG-1S-(K50A), and BAG-1S fused to a heterologous NLS (NLS-BAG-1S) were generated by PCR and are described elsewhere (13, 27).
Effect of Simulated I/R on BAG-1 Expression and Localization in Vitro and in Vivo—We first used Western blotting to determine the effects of simulated I/R on BAG-1 expression in vitro using primary cultures of neonatal rat CMs (Fig. 1a). Cells were subjected to ischemia by incubation for 4 h in ischemic buffer and an atmosphere lacking oxygen. I/R was simulated by subsequent growing cells in a normoxic environment for 4 or 24 h. Rat CMs expressed only the BAG-1L and BAG-1S isoforms, and both proteins were significantly (p < 0.01) increased following simulated ischemia (Fig. 1a). BAG-1L expression was increased (4.2 ± 0.59-fold), and BAG-1S expression was also increased (3.1 ± 0.27-fold) compared with untreated cells (mean of three separate experiments ± S.E.).

Assessment of BAG-1-Mediated Cardioprotection, a Requirement for Cytoplasmic Expression—To determine the importance of elevated BAG-1 expression following I/R, we analyzed the effects of enforced BAG-1 expression on CM apoptosis following simulated ischemia and subsequent reperfusion (Fig. 2). CMs were transfected with expression constructs engineered to overexpress one of the three human BAG-1 isoforms, primary rat CMs were cotransfected with pcDNA3 as a control or with human BAG-1 isoform S, M, or L expression constructs and with an EGFP expression construct to label transfected cells. Cells were subjected to simulated ischemia (gray bars), simulated I/R (black bars), or left untreated as a control (open bars). Apoptosis was analyzed by determining the proportion of TUNEL-positive EGFP-expressing cells.

Nuclear specific BAG-1S construct, BAG-1S NLS, was also used to determine the effect of BAG-1 subcellular localization upon cardioprotection. Error bars represent S.E. from the counting of 200 cells six times per well; data shown are from a representative experiment. * indicates statistically significant changes in apoptosis (p < 0.001) relative to pcDNA-transfected cells.
forms, BAG-1S, -1M, or -1L, at approximately equivalent levels (13, 27). An EGFP expression construct was cotransfected to identify transfected cells, and apoptosis was measured by TUNEL in untreated cells, immediately following 4 h of simulated ischemia or 16 h of reperfusion. In control cells, the proportion of TUNEL positive cells was 9.1 ± 2.2%. Apoptosis was significantly increased following both simulated ischemia and I/R for 42.4 ± 5.0% (p < 0.05 compared with control cells) and 41.3 ± 3.3% (p < 0.001 compared with control cells) of TUNEL-positive cells, respectively. Overexpression of either human BAG-1S or BAG-1M reduced the amount of apoptosis in transfected CM following simulated I/R compared with pcDNA3-transfected cells (22.5 ± 3.1% and 23.5 ± 3.5% respectively; p < 0.001 for both). By contrast, BAG-1L expression did not decrease the percentage of TUNEL-positive myocytes compared with pcDNA-transfected cells (44.0 ± 6%; p > 0.05). Therefore, BAG-1S and BAG-1M, but not BAG-1L, were protective in CM cells following simulated I/R. Although there was a trend for reduced apoptosis following ischemia alone in cells overexpressing BAG-1S or BAG-1M, this failed to reach statistical significance (p > 0.05). However, because the overall levels of apoptosis following ischemia alone are lower than they are following I/R, it is not clear whether this represents a failure to suppress apoptosis under these conditions or that it is more difficult to show a statistically significant change when apoptosis levels are low. Simulated I/R induced only very low levels of CM necrosis, and this was not influenced by overexpression of BAG-1 (data not shown).

We next investigated whether the subcellular distribution of BAG-1S played a significant role in cardioprotection by using a construct that expresses BAG-1S fused to a heterologous NLS (27). Similar to the case of endogenous BAG-1S, overexpressed BAG-1S was localized to both the CM cell nucleus and cytoplasm, whereas NLS-BAG-1, which was expressed at approximately equivalent levels, was localized entirely in the nucleus (data not shown, Ref. 27). In contrast to wild type BAG-1S, NLS-BAG-1S did not prevent apoptosis in transfected CM (Fig. 2), demonstrating that the cytoplasmic localization of BAG-1S is essential for cardioprotection.

Domain Requirements for BAG-1-mediated CM Survival; Cardioprotection Does Not Require BAG-1-Proteasome Binding but Is Dependent upon the Evolutionary Conserved BAG Domain—We next analyzed human BAG-1S deletion and point mutant constructs (13) to identify specific domains important for BAG-1-mediated cardioprotection (Fig. 3). CMs were transfected with mutant expression constructs, and apoptosis was measured following simulated ischemia or I/R. The levels of expression and subcellular distribution of the mutants in CM were identical to that of overexpressed BAG-1S and were unaltered following ischemia and simulated I/R (data not shown). A BAG-1S deletion mutant lacking the evolutionary conserved BAG domain, BAG-1S-(1–155), was unable to protect against apoptotic cell death immediately following I/R (p > 0.05). By contrast, a BAG-1S deletion mutant lacking the ubiquitin-like domain, BAG-1S-(89–230), (refer to Refs. 3, 8, and 13 for a detailed description of the ULD), decreased cell death following I/R to the same extent as wild type BAG-1S did. Similar results were obtained using a BAG-1S point mutant that contained an alanine substitution at Lys-80, which is conserved in the majority of ULD-containing proteins and would be predicted to play an important role in the function of the BAG-1 ULD (5, 13). Therefore, our data demonstrate that the C-terminal BAG domain is obligatory for BAG-1S protection in CM but, unlike in other systems, the N terminus containing the ULD is not required.

Interaction of BAG-1 with HSC70 and RAF-1 in CM and Langendorff-perfused Whole Heart—BAG-1 interacts with multiple cellular proteins, and we performed coimmunoprecipitation experiments to analyze interactions of endogenous BAG-1 with HSC70 and RAF-1, two binding partners thought to be important for the effects of BAG-1 (1–3, 9). Interaction of BAG-1 with HSC70 and RAF-1 was detected in control CM (Fig. 4a). The expression of HSC70 was modestly increased (1.5-fold) following simulated I/R, whereas levels of RAF-1 were unaltered. The interaction between BAG-1 and RAF-1 diminished by >60%, whereas binding between BAG-1 and HSC70 was increased =4-fold (p < 0.01). We also assessed these BAG-1 binding partners in intact heart subjected to simulated I/R by using the Langendorff perfusion apparatus (Fig. 4b).

Consistent with data obtained in CM, the interaction of BAG-1 and HSC70 was increased (>3.5-fold), whereas the binding of BAG-1 to RAF-1 was reduced (4.5-fold) following ischemic stress. Surprisingly, despite these changes in interaction with BAG-1, the expression levels of HSC70 and RAF-1 were unaltered following simulated I/R in intact hearts. Therefore, following I/R there is significantly increased binding of BAG-1 to HSC70 and decreased binding to RAF-1, although the expression levels of these binding partners are altered relatively modestly (CM) or are constant (intact heart).

Functional Assessment of the BAG Domain in Cardioprotection—The BAG domain contains distinct binding sites for HSC/HSP70 and RAF-1, and we therefore generated BAG-1S point mutations to specifically address the role of binding to chaperones in HEK293 cells (Fig. 5a). The BAG domain comprises three α-helices, and we produced mutations of residues within each of the helices, i.e. BAG-1S-(E112A/K116A) (helix 1), BAG-1S-(Q169A/K172A) (helix 2), and BAG-1S-(Q201A/D208A/Q212A) (helix 3). The mutants BAG-1S-(Q169A/K172A) and BAG-1S-(Q201A/D208A/Q212A) have been analyzed previously by Briknarova et al. and prevent binding to chaperones and regulation of the androgen receptor by BAG-1L (7). By contrast, the helix 1 mutations alter conserved charged amino acid residues exposed on the external face of helix 1. The helix mutants were expressed at approximately equivalent levels (Fig. 5b), and, similar to the case with wild type BAG-1, the helix 1 mutant BAG-1S-(E112A/K116A) interacted with HSC70 and HSP70. By contrast, the helix 2 and 3 mutants failed to interact with HSC70 or HSP70 (7). Whereas overexpression of wild type BAG-1S or the helix 1 mutant decreased
apoptosis to the same extent as did the wild type molecule following simulated I/R, the helix 2 and 3 mutants did not protect against apoptosis (p < 0.01 for wild type and helix 1 mutant and p > 0.05, for helix 2 and 3 mutants, respectively) relative to pcDNA-transfected cells (Fig. 5b). These results suggest that binding to the chaperones HSC70 and HSP70 is essential for BAG-1S cardioprotection.

**DISCUSSION**

Here we have demonstrated that BAG-1 has a novel cytoprotective function, i.e. protecting cardiac myocytes from apoptosis induced by simulated I/R. I/R is thought to impose considerable stress on CMs, and, because BAG-1 has been demonstrated to suppress apoptosis in diverse systems (3), it may not seem surprising that BAG-1 also prevents apoptosis induced by I/R in CMs. However, this is the first analysis of BAG-1 function in primary cells, and the mechanism of action revealed by the effects of specific deletion and mutations suggests an unexpectedly distinct mode of action in the heart. It has been suggested that BAG-1 facilitates protein degradation by coordinating the function of chaperones and the proteasome (5); consistent with this suggestion, suppression of stress-induced growth inhibition in breast cancer cells required both the presence of an isolated CM and intact heart. In the intact heart, these changes on the C-terminal BAG domain. In addition to HSC70/ HSP70, the BAG domain also mediates interaction with RAF-1, allowing cells to accumulate in a less proliferative, more apoptosis-resistant state. However, in that case we might have predicted that helix 2 and 3 mutants deficient in chaperone binding of HSC70/HSP70 and RAF-1 is competitive (9), and if one binding might be more active than the wild type molecule. An alternate explanation for the role of BAG-1/RAF-1 in stress-induced apoptosis is that increased HSC70 levels lead to a decrease in RAF-1 activity by sequestering BAG-1, possibly preventing cells from accumulating in a less proliferative state. However, in that case we might have predicted that increased BAG-1 expression would maintain RAF-1 activity, thereby promoting cell death. Interestingly, following simulated I/R, there is an increase in binding of BAG-1 to HSC70 but a decrease in binding to RAF-1 in both isolated CM and intact heart. In the intact heart, these changes in associations of BAG-1 are independent of changes in the proteasome does not appear to play a critical role in BAG-1-mediated cell survival. (It remains possible, however, that the ULD is required for suppressing apoptosis associated with ischemia alone (Fig. 2), because there was a trend for decreased apoptosis in cells overexpressing wild type BAG-1S but not BAG-1S–(89–230) or BAG-1S–(K80A) at this point. However, the levels of apoptosis induced at this time were modest, and these differences did not reach statistical significance.)

Similar to the situation with many other systems (7, 13), however, BAG-1-mediated cytoprotection in CM was dependent on the C-terminal BAG domain. In addition to HSC70/ HSP70, the BAG domain also mediates interaction with RAF-1, but our overexpression studies demonstrate that binding to chaperones is essential for BAG-1-mediated cytoprotection. Consistent with this finding, chaperone function is required for optimal cell survival in cells treated with apoptotic stimuli. The binding of HSC70/HSP70 and RAF-1 is competitive (9), and if BAG-1 were promoting survival via activation of RAF-1-mediated anti-apoptotic signaling pathways, we would therefore have predicted that helix 2 and 3 mutants deficient in chaperone binding might be more active than the wild type molecule. An alternate explanation for the role of BAG-1/RAF-1 in stress-induced apoptosis is that increased HSC70 levels lead to a decrease in RAF-1 activity by sequestering BAG-1, possibly allowing cells to accumulate in a less proliferative, more apoptosis-resistant state. However, in that case we might have predicted that increased BAG-1 expression would maintain RAF-1 activity, thereby promoting cell death. Interestingly, following simulated I/R, there is an increase in binding of BAG-1 to HSC70 but a decrease in binding to RAF-1 in both isolated CM and intact heart. In the intact heart, these changes in associations of BAG-1 are independent of changes in the proteasome does not appear to play a critical role in BAG-1-mediated cell survival. (It remains possible, however, that the ULD is required for suppressing apoptosis associated with ischemia alone (Fig. 2), because there was a trend for decreased apoptosis in cells overexpressing wild type BAG-1S but not BAG-1S–(89–230) or BAG-1S–(K80A) at this point. However, the levels of apoptosis induced at this time were modest, and these differences did not reach statistical significance.)

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expression of either HSC70 or RAF-1, suggesting that posttranslational mechanisms controlling binding of BAG-1 to various interaction partners exist.

The BAG domain is sufficient to modulate chaperone activity, but results of studies investigating the effects on chaperone function are inconsistent. In some systems, BAG-1 suppresses chaperone-mediated refolding, whereas in others it is stimulated (1–3). The effect of BAG-1 is likely to be very dependent on the relative concentrations of HSC70/HSP70 and other co-chaperones, such as HSP40, which modulate the chaperone ATPase cycle. Thus, in CMs it is possible that BAG-1 stimulates protein refolding to protect cells from I/R-induced apoptosis via the BAG domain alone, whereas in cancer cells BAG-1 may function by coupling chaperone function to the proteasome. Notably, cancer cells are particularly sensitive to inhibition of the proteasome, suggesting that its role is altered in malignant cells (14, 28). Although studies of refolding have been performed using luciferase and β-galactosidase, these two are clearly not physiologic substrates. Determining the role of refolding will require identification of endogenous substrates in the two systems.

Further mechanistic differences are revealed by our analysis of the activity of specific isoforms and their subcellular localization. First, in breast cancer cells exposed to heat shock (13) or cervical carcinoma cells treated with staurosporine (29), BAG-1S and BAG-1L were equally effective in the suppression of growth inhibition/apoptosis. Although initially differentially localized, BAG-1S (and HSC70/HSP70) relocalized to the nucleus within 2–4 h following heat shock in these cells. Taken together, these data suggest a common site of action for BAG-1 isoforms in the nucleus of cancer cells. By contrast, only BAG-1S suppressed cell death in CM, and this was dependent on its cytoplasmic localization, because tagging BAG-1S with a heterologous NLS to anchor it to the nucleus abrogated its survival functions. In CM, BAG-1 localization appears to be relatively complex and dynamic with both BAG-1S and BAG-1L partitioning between the cytoplasm and the nucleus. However, following simulated ischemia, only BAG-1L, and not BAG-1S, relocalized to the nucleus. These data point to a critical cytoplasmic site of action for BAG-1 in CMs.

Taken together, our results suggest that the induction of BAG-1 following ischemia represents a cellular survival response that is important for limiting subsequent cell death. In CMs, we propose that elevations in BAG-1 and HSC70 expression following ischemia leads to increased abundance of cytoplasmic BAG-1/HSC70 complexes, which appear to play a critical role in protecting cells from I/R-induced apoptosis. Induction of BAG-1 during simulated I/R injury is concomitant with that of heat shock proteins HSP70 and HSC70, which, in earlier work from our laboratory, also proved to be cardioprotective (30). However, in contrast to previous studies, BAG-1 function in CM appears to be dependent on a cytoplasmic activity that is independent of the proteasome, thus providing evidence that BAG-1 can influence cell survival by multiple mechanisms that may be cell type- or stimuli-dependent. Because our data indicate that BAG-1 mediates cardioprotection through binding to HSP70/HSC70, we postulate that acute ischemic stresses (such as transient ischemia associated with angina), which are potentially capable of raising the basal levels of both BAG-1 and chaperone proteins, may provide in cardiac cells a synergistic mechanism of protection against ischemia/reperfusion and play a role in the modulation of cardiac preconditioning. Our results suggest mechanistic differences in CMs that could be preferentially targeted to manipulate cardiac-specific pathologies based around apoptosis, such as, for example, chronic hypertrophy and myocardial infarction.

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