Overexpression of HAX-1 Protects Cardiac Myocytes From Apoptosis Through Caspase-9 Inhibition

Yuchi Han, Yee-Shiuan Chen, Zhilin Liu, Natalya Bodyak, Debra Rigor, Egbert Bisping, William T. Pu, Peter M. Kang

Abstract—Caspase-9 is a critical regulator of mitochondria-mediated apoptosis. We found that adult cardiac myocytes, but not nonmyocytes, have high caspase-9 expression, and exhibit relative resistance to caspase-9–induced cell death. Thus, we hypothesized that cardiac myocytes possess factors that resist apoptosis. Through a yeast two-hybrid screening of adult human heart cDNA library, we identified HS-1 associated protein-1 (HAX-1), a 35-kD BH-domain containing protein localized to the mitochondria as one of the molecules that interacts with caspase-9. Recombinant HAX-1 protein inhibited caspase-9 processing in a dose-dependent manner in a cell-free caspase activation assay. Overexpression of HAX-1 in adult cardiac myocytes conferred 30% protection from apoptosis as compared with the control. Suppression of HAX-1 expression using siRNA-HAX-1 resulted in significant cell death in adult cardiac myocytes, suggesting the importance of HAX-1 in cardiac myocyte resistance to apoptotic stimulation. On apoptotic stimulation, some caspase-9 translocated to the mitochondria and co-localized with HAX-1, confirming the spatial proximity of caspase-9 and HAX-1. In summary, HAX-1 is a newly identified anti-apoptotic factor and its mechanism of action is through caspase-9 inhibition. (Circ Res. 2006;99:415-423.)

Key Words: apoptosis | cardiac myocytes | caspase | HAX-1 | mitochondria

Apoptosis is a form of cell death with critical functions in the development of the organism and in maintaining cellular homeostasis throughout life. A key regulatory player in the mitochondria-mediated apoptosis pathway is caspase-9. On apoptotic stimulation, cytochrome c is released from the mitochondria to the cytosol, where, in the presence of dATP, it forms an apoptosome complex with apoptotic protease–activating factor-1 (Apaf-1) and caspase-9. Apoptosome complex formation activates caspase-9, which then proteolytically activates caspase-3, one of the principal proteases participating in the execution of cell death.

Multiple studies have shown that the mitochondrial apoptosis pathway is activated in cardiac myocytes in response to a variety of stimuli, including oxidative stress, suggesting the importance of caspase-9 in regulating apoptosis in heart. Several mechanisms are known to be involved in regulating caspase-9. To date, the only direct caspase-9 inhibitors that have been identified are the inhibitor of apoptosis proteins (IAPs), which comprise a family of evolutionarily-conserved, homologous proteins first discovered in baculovirus. Investigations into other mechanisms controlling caspase-9 activation have largely centered around the prevention of cytochrome c release from mitochondria as orchestrated by the pro- and anti-apoptotic members of the Bcl-2 protein family. Additional mechanisms, however, now include the recently discovered protein, Aven. Aven is highly expressed in the heart and skeletal muscle and interacts with Apaf-1 and Bcl-2 family proteins to prevent the activation of the apoptosome by interfering with Apaf-1 self association. Caspase-9 is also inactivated by Akt (serine/threonine kinase) phosphorylation of procaspase-9. And finally, it has been proposed that lack of Apaf-1 is yet another mechanism promoting resistance to apoptosis in cardiac myocytes.

Adult cardiac myocytes are highly differentiated cells with a very limited ability to proliferate and a very low level of baseline apoptosis. We show, however, that even though the heart is characterized by a high level of caspase-9 expression, cardiac myocytes are resistant to caspase-9–induced apoptosis. It is probable, therefore, that cardiac myocytes also possess an effective caspase inhibitor. In this report, we sought candidate molecules by screening a human heart cDNA library with a yeast two-hybrid system to identify proteins that interact with caspase-9. We identified HS-1 associated protein (HAX-1), a 35-kDa intracellular protein, as one of the proteins that interact with caspase-9. HAX-1 mRNA is ubiquitously expressed in all tissues. HAX-1, a known mitochondrial protein, contains BH1 and BH2 domains and, although the BH3 domain is absent, it has some similarity to Nip3, a pro-apoptotic mitochondrial protein. In this study, we demonstrate that HAX-1 is an anti-apoptotic...
molecule, and that it protects cardiac myocytes from hypoxia/reoxygenation-induced apoptosis by inhibiting caspase-9.

Materials and Methods

Materials

Materials used are from the following sources. Antibodies: HAX-1 (Transduction Laboratories), caspase-9 (Pharmergen), GAPDH (BD Biosciences), cytochrome oxidase subunit IV (Molecular Probes), and anti-mouse HRP and anti-rabbit HRP (Jackson Immuno Research Laboratory). MitoTracker Red CMXRos (Molecular Probes), FITC-Annexin V kit (Zymed). Homozygote mouse embryonic fibroblasts (MEFs) lacking caspase-9 (C9KO MEFs), caspase-9, and caspase-3 cDNA were generous gifts from Dr K. Kuida (Vertex Pharmaceuticals).

Cell Culture

Adult rat cardiac myocyte (ARCM) cultures were obtained from the hearts of female Sprague-Dawley rats according to a previously published protocol.4 Apoptosis was induced by hypoxia/reoxygenation (H/R) according to a previously published protocol. Cell survival was measured using an MTT assay (Sigma, St. Louis, Mo) and annexin V staining.

Immunocytochemistry

Immunofluorescent staining was performed according to the published protocol, and images acquired using confocal microscopy (BioRad). When MitoTracker was used, it was added to the cell culture for 30 minutes before fixation. Immunoprecipitation was performed according to the published protocol.

Yeast Two-Hybrid Screen

Yeast two-hybrid screening was performed using MATCHMAKER Two-Hybrid System 3 as recommended by the manufacturer’s protocol (Clontech). Briefly, wild-type human caspase 9 and mutant caspase 9 (with a cysteine to alanine substitution in the catalytically active site) fused to GAL4 DNA-BD were used as baits and transformed separately into strain AH109 yeast. Each of the two mating cultures was then grown under high stringency conditions. Identified clones were amplified and sequenced. Identified clones were amplified and sequenced. pACT2 HAX was subcloned into the pcDNA3.1 expression vector for further study.

Recombinant Protein Synthesis and Purification

GST tagged pro-caspase-9 was synthesized by cloning procaspase-9 cDNA into pESP-3 (Strategene) with a C-terminal GST tag. The segment of procaspase-9-GST was then cloned into a protein expression vector, prSET (Invitrogen), and expressed in BL21 Escherichia coli (Invitrogen) with 0.1 mmol/L IPTG (Sigma) stimulation. The fusion protein was purified by GST column (Amersham Biosciences). Human HAX-1 cDNA was cloned into the prSET vector and expressed in BL21 E. coli with IPTG stimulation. The His6-tagged HAX-1 recombinant protein was purified on the TALON column according to the manufacturer’s protocol (Clontech). Purified protein concentration was measured using the bicinechonic acid (BCA) method.

Caspase Activation Analysis

Cell-free caspase activation analysis was performed with 35S-labeled procaspase-9 and procaspase-3 proteins prepared by in vitro translation using TntC Coupled Reticulocyte Lysate System (Promega). Cell-free caspase activation was performed according to the published protocol. A substrate cleavage assay for caspase-9 using LEHD-pNa as a substrate was performed according to the published protocol.

Recombinant Adenoviruses

HAX-1 cDNA was cloned into pShuttle-CMV vector (Invitrogen) using HindIII and Pmel. The recombinant adenovirus expressing HAX-1 was generated using the pAdEasyRecombinant adenovirus vector system according to the manufacturer’s protocol (Strategene). For inducible caspase-9 recombinant adenovirus, we used the Adeno-X Tet-off Gene Expression System according to the manufacturer’s recommendation (Clontech). The virus was purified using a CsCl2 gradient. The viral titer was determined using the Tissue Culture Infectious Dose 50 Method (QbioGene). All experiments were performed using 50 multiplicity of infection (moi) to transduce the cells.

RNAi Adenovirus

Single-stranded sense siRNA and anti-sense siRNA oligo sequences were cloned downstream of the H1 RNA polymerase III promoter in pENTR-H1/Zeo, an RNAi expression vector in which the correct recombinants are conveniently selected for by Zeocin resistance and in which the RNAi expression cassette is flanked by recognition sequences for the Gateway recombination system (Invitrogen). LR clonase was used to transfer the RNAi expression cassette to pAd/gateway; a derivative of Adeno-X (Clontech) containing a gateway destination cloning cassette. The recombinant adenovirus containing the siRNA expression cassette was transfected into 293K cells. The HAX siRNA2 sequence used was 5’-ACCAAGATCTACTAACCGAT-3’, which is located in the coding region (640 to 660 bp). Control siRNA (siRNAc) was a scrambled siRNA not having any sequence match in rat or mouse genomes.

Subcellular Fractionation

Subcellular fractionation was done according to the published protocol. The cells were homogenized using Down’s homogenizer followed by serial centrifugation at 1000g for 20 minutes (pellet=nuclei and unbroken cells), at 10,000g for 20 minutes (pellet=mitochondria), and at 10,000g for 60 minutes (supernatant=cytosolic S100 fraction). Cytochrome oxidase IV was used as a specific mitochondria marker.

Generation and Analysis of HAX-1 Mutants

Deletion mutants were generated by RT-PCR using a selection strategy based on the structural domains of HAX-1. HAX-1 mutants were generated from the full-length cDNA sequence of HAX-1, which contains 840 nucleotides (a 279 amino acid sequence). The anti-apoptotic effect of overexpression of HAX-1 mutants was studied by transient co-transfection of each HAX-1 mutant with GFP vector in COS cells. After 24 hours, caspase-9 was overexpressed using AdCaspase-9. The number of GFP-positive cells was determined 36 hours after AdCaspase-9 infection. The control group was transfected with empty pcDNA and GFP vectors.

Statistical Analysis

All data were expressed as mean±SEM. Between groups and among groups comparisons were conducted with unpaired Student t tests and ANOVAs, respectively. P<0.05 was considered significant.

Results

Cardiac Myocytes Are Resistant to Caspase-9 Induced Apoptosis

To characterize the distribution of caspase-9, we determined the protein expression level of caspase-9 in various tissues. We observed high levels of caspase-9 in heart, brain, and skeletal muscle (Figure 1A). This was a somewhat unexpected finding, because all three are highly differentiated organs that are expected to be relatively resistant to apoptosis. We, next, examined caspase-9 expression in the heart at three different developmental stages. The embryonic heart proved

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to have a relatively low level of caspase-9, whereas levels were significantly greater in neonatal and adult heart (Figure 1B). In addition, we compared the expression of caspase-9 in dissociated cardiac myocytes and non-myocytes from the same heart. We found that caspase-9 was predominantly present in cardiac myocytes (Figure 1C).

To determine the effects of caspase-9 overexpression in cardiac myocytes, we used a recombinant adenovirus system. The construction of constitutive overexpression of caspase-9 was not feasible because transfection of the adenovirus vector in HEK293 cells, the cells that are needed to generate and expand adenovirus, resulted in complete HEK293 cell death (unpublished observations). Instead, we generated recombinant adenovirus with tetracycline-inducible expression of wild-type caspase-9 (AdCasp9) or the dominant-negative mutant form of caspase-9, which has an active cysteine site substituted with alanine (AdmCasp9) (Figure 2A). The expression of caspase-9 was titer-dependent, with significantly increased expression at 10 to 100 moi. In addition, caspase-9 expression was completely suppressed at doxycycline concentrations of >1 ng/mL. Overexpression of caspase-9 in non-myocytes, not surprisingly, resulted in >70% cell death (Figure 2B, left panel). However, unexpectedly, when caspase-9 was overexpressed in ARCM culture using AdCasp9, there was no significant cell death compared with the control adenovirus expressing lacZ (AdLacZ) or noninduced AdCasp9 infection (Figure 2B, right panel). Overexpression of mCasp9 resulted in the complete attenuation of cell death in non-myocytes, confirming that the previous finding was attributable to specific caspase-9 activity (Figure 2B).

Caspase-9 is initially produced as a pro-caspase-9 (~47 kDa), and undergoes processing into the activated caspase-9 (~37 kDa). To determine the mechanism of resistance of cardiomyocytes to caspase-9 induced apoptosis, we first examined whether caspase-9 is activated in this system using Western blot analysis to reveal the presence of caspase-9 processing. This experiment showed that caspase-9 is not activated in ARCM; in contrast, non-myocytes from the same heart showed full activation (Figure 2C). To confirm that the processing of pro-caspase-9, as seen in our Western blots, translates to the activation of caspase-9 in non-myocyte, we performed a substrate cleavage assay using LEHD-pNa as a caspase-9 substrate. Corresponding to the caspase-9 processing finding, we observed significant increase in caspase-9 activity in non-myocyte, but not in ARCM, after transducing with AdCasp9 (Figure 2D). These data suggest that cardiac myocytes possess factor(s) that inhibit the processing of caspase-9 to an activated form and thus provide apoptotic resistance.

**HAX-1 Is Identified as a Caspase-9 Inhibitor**

To identify proteins that interact with caspase-9, we used a yeast two-hybrid system. Wild type caspase-9 and the dominant-negative mutant of caspase-9 were used as bait to screen a human adult heart cDNA library. We identified 32 genes (24 known and 8 unknown) that interact with caspase-9 using the yeast two-hybrid system. Wild type caspase-9 and the dominant-negative mutant HAX-1 protein inhibits the processing of caspase-9 as well as caspase-3 in a concentration-dependent manner (Figure 3D). We speculate that inhibition of pro-caspase-3 processing is most likely conveyed through inhibition of the activation of caspase-9 in the S100 fraction. However, the possibility of HAX-1 directly interacting with, and thereby inhibiting, caspase-3 cannot be excluded. These findings suggest that HAX-1 is a novel inhibitor of caspases that exerts its effect by directly interacting with, and inhibiting, caspase-9 processing.
HAX-1 Protects Against H/R-Induced Cell Death

The next studies were conducted to determine if HAX-1 is able to protect cardiac myocytes from apoptotic stimulation. Overexpression of HAX-1 in cardiac myocytes using HAX-1 recombinant adenovirus resulted in significant attenuation of H/R-induced cell death (control normoxia 1.00 ± 0.01, control H/R 0.77 ± 0.06, control + H/R versus HAX-1 + H/R; P < 0.05) (Figure 4A). The improved cell survival was predominantly caused by apoptosis as demonstrated by Annexin V staining of the ARCM (Figure 4B). Furthermore, there was a significant activation of endogenous caspase-9, as seen by an increased processed caspase-9 band during H/R in ARCM. The activation of endogenous caspase-9 by H/R was significantly attenuated by HAX-1 overexpression in ARCM (Figure 4C and 4D). These findings are consistent with our previous data from a cell-free system indicating that HAX-1 protection is conferred through caspase-9 inhibition.

In addition, we investigated the effect of knockdown of HAX-1 gene products in cardiac myocytes using HAX-1–specific siRNA. We generated three recombinant adenoviruses expressing specific siRNA targeting sequences from three different regions of HAX-1. All three showed significant knockdown of endogenous HAX-1 mRNA expression (Figure 4E). We chose AdsiRNA for subsequent experiments. During normoxia, we found that infecting with AdsiRNA2, thus resulting in HAX-1 expression knockdown, resulted in significantly decreased cell survival in ARCM compared with the control AdsiRNAc expressing a scrambled sequence (Figure 4E). In fact, infecting with AdHAX-1 was not able to rescue AdsiRNA2-induced cell death, which is likely secondary to attenuation of HAX-1 overexpression. During H/R, HAX-1 overexpression protected cells from H/R-induced apoptosis in cells expressing control virus AdsiRNAc. However, overexpression of siRNA2 obliterated the protective effect of HAX-1 during H/R-induced apoptosis (Figure 4B and 4F). This effect is most likely caused by the fact that siRNA2 significantly knocks down endogenous HAX-1 expression and prevents overexpression of HAX-1, thereby neutralizing the effect of anti-apoptotic effect of HAX-1 during H/R.

Identification of HAX-1 Structural Domain and Anti-apoptotic Activity

We further defined the structural domains involved in the interaction between HAX-1 and caspase-9, and studied whether some or all of them are necessary for the anti-apoptotic activity of HAX-1. To address this issue, we generated and examined various HAX-1 deletion mutants based on known or putative domains in HAX-1 (Figure 5A). The mutants included deletion of the following domains: Δ56 (56 amino acids (AA) includes the BH1 domain), Δ89 (89 AA includes the BH1/BH2 domains), Δ128 (128 AA includes the BH1/BH2/PEST domains), Δ174 (174 AA includes the BH1/BH2/PEST domains), Δ205 (205 AA includes the BH1/BH2/PEST domains), ΔTMD+ (56 N-term AA and 73AA C-term including the TM domain), and ΔTMD (18AA includes the TM domain). We found that HAX-1 mutant Δ205, which lacks the N-terminal 205 amino acids, did not bind with caspase-9 (Figure 5B through 5D), suggesting that the 32-amino acid domain (175–206) is required to bind caspase-9. To confirm that 175 to 206 domain is the caspase-9 binding domain, we further generated three more mutants: 1 to 129
knockout mouse (C9KO MEF) (generous gift from Dr Keisuke Kuida), which completely lacks caspase-9. In wild-type MEF, HAX-1 significantly inhibited H$_2$O$_2$-induced apoptosis, although not complete (Figure 5F). However, in C9KO MEF, there was complete elimination of apoptosis after H$_2$O$_2$ stimulation. Overexpression of HAX-1 did not have significant effect on H$_2$O$_2$-induced apoptosis in C9KO MEFs, suggesting that effect of HAX-1 is predominately attributable to inhibition of caspase-9.

**Mitochondrial Caspase-9 Increases on H/R**

Caspase-9 and HAX-1 are shown to be expressed predominantly in cytosol and mitochondria, respectively. Consistent with previous reports, our initial study also showed HAX-1 and caspase-9 predominantly localized to mitochondria and cytosol in ARCM, respectively (Figure 6A and 6C). To determine subcellular localization of HAX-1 and caspase-9 during apoptotic stimulation, we performed triple immunofluorescent labeling for HAX-1, caspase-9 and mitochondria after H/R. We found that in ARCM exposed to H/R, there was increased co-localization of HAX-1 and caspase-9 in mitochondria (Figure 6B). For example, there was increased triple staining of caspase-9, HAX-1 and mitochondria, shown as white color after H/R (green, blue and red combination) compared with the double staining of HAX-1 and mitochondria, shown as magenta (blue and red combination). Furthermore, subcellular fractionation during normoxia and hypoxia/reoxygenation showed partial translocation of caspase-9 into mitochondria (Figure 6C). These findings suggest that on apoptotic stimulation, some caspase-9 translocates to mitochondria and interacts with HAX-1 inside the mitochondria.

**Discussion**

HAX-1 was discovered in 1997, and subsequently was shown to interact with a diverse group of molecules: hematopoietic lineage specific protein-1 (HS-1)$_{19}$; inflammatory cytokine, interleukin-1$_{15}$; polycystic kidney disease protein PKD2$_{26}$; EBV nuclear antigen 527; inflammatory cytokine, interleukin-125;3 polycystic kidney disease protein PKD2,26 EBV nuclear antigen 527;3 untranslated region of human vimentin mRNA28; and K15 protein of Kaposi sarcoma-associated herpesvirus.29 Although these reports suggest HAX-1 interacts with cytoskeletal elements and viral proteins, the biological significance of these interactions remains unclear. A number of investigators have also found overexpression of HAX-1 in Jurkat or Hela cell lines to be anti-apoptotic under Fas treatment,19 and Bax-induced apoptosis,29 offering evidence that HAX-1 executes its anti-apoptotic function by interacting directly with caspase-9 and inhibiting caspase-9 processing and activation. Also, we showed that HAX-1 does so at an early stage of apoptosis and that the interaction may...
also occur within the mitochondria. These results are congruent with other recent information concerning the regulation of HAX-1 by Omi/HtrA2 protease during cell death, which describes HAX-1 degradation occurring early in the process, at a time when Omi is still located in the mitochondria and before it translocates to the cytosol to degrade IAPs.

Our results offer not only a new mechanism by which HAX-1 functions as an endogenous anti-apoptotic molecule,
but also a new mechanism by which caspase-9 is inhibited. The only previously known direct inhibitors of caspase-9 are the IAPs.8,9 XIAP interacts with caspase-9 in the cytosol by binding to the active caspase-9-Apaf-1 holoenzyme complex through the amino terminus of the linker peptide on the small subunit of caspase-9, exposed after proteolytic processing at Asp315.8 HAX-1 has BH1, BH2, and a transmembrane domain (TMD).19 The TMD contains a hydrophobic COOH-terminal, which tail anchors the protein to the mitochondrial outer membrane.31 Our data indicates that amino acid sequence between 174 to 206 is required for HAX-1 binding to caspase-9. To explore the significance of this sequence, we aligned HAX-1 sequence 174 to 206 against the protein motif database BLOCKS (blocks.fhcrc.org) using the default parameters. Interestingly, this analysis yielded one possible hit corresponding to ClassID Cytochrome C Signature (IPB002324D). This motif is defined by several Pro residues around the sixth ligand Met and a conserved Trp residue near the C-terminus; the membership is shared by cytochrome molecules from several bacterial species.

Multiple lines of evidence indicate that active caspase-9 is partially localized within mitochondria.32–35 This evidence...
ties as a specific inhibitor of caspase-9 activation through its anti-apoptotic property. However, from this study, we cannot rule out the possibility of intermediary molecule(s) that may be involved in this mechanism.

In summary, we have identified HAX-1 as a protein that has two sources: evidence for mitochondrial caspase-9 translocating to the cytosol on the induction of apoptosis, and evidence that in early apoptosis active caspase-9 translocates from cytosol to mitochondria, and then back to cytosol. Our present data that show that mitochondrial caspase-9 increases in response to the apoptotic stimulus adds to the evidence for mitochondrial caspase-9 translocation during normoxia and after H/R in ARCM. COX IV was used for a mitochondria-specific control marker.

![Figure 6. Subcellular localization of HAX-1 and Caspase-9 in ARCM. A, Immunofluorescent staining showing the subcellular localization of HAX-1 (green), MitoTracker (red). Caspase-9 did not co-localize with MitoTracker (bottom, right). B, Triple immunofluorescent staining showing caspase-9 translocation to mitochondria and interacting with HAX-1. In ARCM exposed to H/R, some caspase-9 co-localized with HAX-1 in mitochondria. Caspase-9 (green), mitochondria (red), and HAX-1 (blue). C, Caspase-9 subcellular redistribution during normoxia and after H/R in ARCM. COX IV was used for a mitochondria-specific control marker.](image-url)

direct interaction with caspase-9. In addition, knock down of HAX-1 gene products using siRNA demonstrated the essential function of HAX-1 in survival. Further characterization of HAX-1 is needed to better elucidate the molecular mechanism of its anti-apoptotic effects in heart, and the functional significance of its caspase-9 inhibition in vivo.

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**Disclosures**

None.

**References**


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