**Competition through Dimerization between Antiapoptotic and Proapoptotic HS-1-associated Protein X-1 (Hax-1)**

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Background: Hax-1 is a family of apoptotic regulators. The prototypical variant 1 has an antiapoptotic function.

Results: Hax-1 variant 2 promotes cell death and can abrogate the protective effect of variant 1.

Conclusion: The ratio of anti- and proapoptotic Hax-1 variants is likely to determine cell fate via sequestration or inactivation.

Significance: Different splice variants of Hax-1 have opposing roles in regulating apoptosis.
and v4 abrogates the protective and prodeath effects of v1 and v2/v4, respectively, via regulation of cytochrome c release. This is modulated by the formation of homo- and heterotypic dimers of Hax-1 proteins. Therefore, our findings document, for the first time, that Hax-1 comprises a family of antiapoptotic and proapoptotic proteins that may regulate cell fate under stress conditions via the formation of homo- or heterodimers.

**EXPERIMENTAL PROCEDURES**

**Myocardial Infarction**—Frozen lypohelated heart tissue from adult Sprague-Dawley rats was donated by Dr. William Stanley (University of Maryland, School of Medicine). Heart failure was induced by constriction of the left coronary artery via ligation to simulate myocardial infarction, as described previously (20, 21). The ligation clamp remained in place for 12 weeks. Control animals were as follows: rat v1 and v2, 5’-ACTGGAGCTCATGAGGCTTTCGTGGTCTCATGGTCTTTTGGTAT-3’ (forward) and 5’-ACTGGAGCTCATGAGGCTTTCGTGGTCTTTTGGTAT-3’ (reverse). For human v1, the above rat forward primer was used with reverse primer 5’-ACTGGCCGGGTGATCCGGGACCCGAACAC-3’.

**Quantitative RT-PCR**—Poly-A mRNA was isolated from rat cardiac tissue subjected to myocardial infarction or sham surgery using the MicroPoly(A) Purist kit (Ambion, Life Technologies, Grand Island, NY). Quantitative RT-PCR reactions were set up in 50 μl of volume using Bio-Rad iScript and IQ SYBR Green Supermix (Bio-Rad). Hax-1 variant-specific primers were designed to span exon/intron junctions with an optimal Tm of 60 °C and a length of between 18 and 25 bases. The seven known rat Hax-1 variants, we were able to specifically amplify variants 1, 2, 4, and 6. The primers used were designed according to the published rat variant sequences (accession numbers NM_181627.2, AY919342.1, and AY291064.2) and were as follows: v1, 5’-GACCTCGGAGCCACAGAGATC-3’ (forward) and 5’-CCTGGAAGTTTACGAGATGGGG-3’ (reverse); v2, 5’-GACCTCGGAGCCACAGAGATC-3’ (forward) and 5’-CCTGGAAGTTTACGAGATGGGG-3’ (reverse); v4, 5’-GCAAGTATGTGAGGCACAGAGATC-3’ (forward) and 5’-CCTGGAAGTTTACGAGATGGGG-3’ (reverse); v5, 5’-CCTGGAAGTTTACGAGATGGGG-3’ (forward) and 5’-CCTGGAAGTTTACGAGATGGGG-3’ (reverse).

**Western Blotting**—Immunoblot analyses were performed as described previously (19). The only modification to this protocol was that 25 mg of heart tissue was homogenized in Nonidet P-40 buffer containing 10 mM NaPO4, 2 mM EDTA, 10 mM NaN3, 0.9% NaCl, and 2% Nonidet P-40 in the presence of a protease inhibitor mixture (Roche, catalog no. 11697498001) using a tissue grinder (VWR, Radnor PA, model no. 47747-366). Protein lysates from three independent experiments were prepared and analyzed. The following primary antibodies were used: Hax-1 (1:1000 dilution, BD Biosciences), His (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX), α tubulin (1:1000 dilution, Sigma-Aldrich, St. Louis, MO), cytochrome c (1:200 dilution, Abcam, Cambridge, MA), and GAPDH (1:1000 dilution, Santa Cruz Biotechnology). This was followed by goat anti-mouse or donkey anti-rabbit secondary antibody (1:2500 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Blots were developed using an alkaline phosphatase-based chemiluminescent system (Applied Biosystems, Foster City, CA). Relative levels of immunoreactive bands were quantified using ImageJ software and densitometric analysis (National Institutes of Health, Bethesda, MD).

**Generation of Overexpression Plasmids**—Overexpression constructs were generated by cloning the complete coding sequence of rat Hax-1 v1 and v2 and human Hax-1 v1 and v4 (accession numbers: NM_181627.2, AY919342.1, NM_006118.3, and EU190982.1, respectively) into the pIRE2-ZsGreen1 or pIRE2-ZsRed1 vector (Clontech, Mountain View, CA) using the Sac1 and Xma1 restriction sites. The primes used were as follows: rat v1 and v2, 5’-ACTGGAGCTCATGAGGCTTTCGTGGTCTTTTGGTAT-3’ (forward) and 5’-ACTGGCCGGGTGATCCGGGACCCGAACAC-3’ (reverse). For human v1, the above rat forward primer was used with reverse primer 5’-ACTGGCCGGGTGATCCGGGACCCGAACAC-3’.

**Cell Culture and Transfections**—HEK293 cells (ATCC) were cultured in growth medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) and maintained in a water-jacketed 5% CO2 incubator at 37 °C. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer, followed by medium change 5 h after transfection.

**Measurement of Cell Viability using the XTT Assay**—3 × 105 HEK293 cells were seeded in standard 6-well plates 24 h prior to transfection. 24 h after transfection, plasmid expression was confirmed by visualization of GFP or red fluorescent protein fluorescence. Cells were treated with either 2 μM thapsigargin for 24 h, 4 μM calcimycin for 24 h, or 0.33 mM H2O2 for 4 h. All treatments were performed in sodium pyruvate-free DMEM in the absence of serum or antibiotics. Cell viability was measured using XTT sodium salt (2,3-Bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide inner salt) (Sigma-Aldrich) according to the instructions of the manufacturer.

**Evaluation of Apoptosis via FACS Analysis**—Cells were plated, transfected, and treated with the indicated chemicals as for the XTT assay. Following treatment, cells were collected using 0.025% trypsin (Invitrogen) and prepared for FACS analysis using the annexin V phycoerythrin apoptosis detection kit (BD Biosciences) according to the protocol of the manufacturer. FACS analysis was performed on a BD FACS Scan Analyzer by the University of Maryland Flow Core staff. Only GFP-positive, red fluorescent protein-positive, or GFP/red fluorescent protein-positive cells were included in our analysis to ensure that only transfected cells were analyzed.

**Production of Recombinant Hax-1 Proteins**—His6- or GST-tagged recombinant proteins were generated for use in binding assays. Full-length rat Hax-1 v1 and v2 were cloned into the pet30a (Novagen, Darmstadt, Germany) and pGEX (GE Life Sciences, Pittsburgh, PA) vectors for expression of His6- and GST-tagged fusion proteins, respectively. Proteins were expressed in BL21 DE3 cells following standard techniques. For protein extraction, bacterial pellets from a 100-ml culture were resuspended in 20 ml STE buffer (10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA (pH 7.5)). After one freeze-thaw cycle,
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Lysozyme was added to the cell pellet to a final concentration of 100 μg/ml, followed by incubation on ice for 15 min. Subsequently, 5 mM DTT and 1% N-lauroylsarcosine were added to the cell slurry, followed by sonication for four 10-s bursts on medium power and centrifugation at 8900 × g at 4 °C for 30 min. The supernatant containing the recombinant protein of interest was removed, and 2% Triton X-100/20 mM CHAPS were added, followed by incubation with gentle rocking for 4 h at 4 °C. All samples were dialyzed in cold PBS in the presence of 10 mM NaNO3 and 1 mM EDTA. His6-tagged proteins were purified using nickel-nitrioltriacetic acid beads (Qiagen, Hilden, Germany) and gravity flow columns, following the protocols of the manufacturer. GST-tagged proteins were incubated with glutathione-Sepharose beads (Novagen) according to the instructions of the manufacturer.

Protein Binding via GST Pulldown—GST-tagged rat Hax-1 v1 was bound to glutathione-Sepharose beads and incubated with 3 μg of His-tagged Hax-1 v1 or v2 overnight at 4 °C in pulldown buffer (50 mM Tris (pH 7.5), 120 mM NaCl, 10 mM NaNO3, 2 mM DTT, and 0.5% Tween). Beads were washed five times with wash buffer (PBS with 10 mM NaNO3 and 0.1% Tween), and bound proteins were eluted with 2× lithium dodecyl sulfate buffer (Invitrogen), followed by boiling at 95 °C for 10 min and separation on a 4–12% bis-Tris gel. Our standard Western blotting procedure was followed, using a His6 tag antibody (catalog no. SC-803, Santa Cruz Biotechnology).

Protein Binding via Surface Plasmon Resonance—Surface plasmon resonance was performed using a BiaCore 3000 instrument as described previously (22, 23). Analyte concentration ranged from 25–500 nM. Experiments were performed independently at least three times, yielding similar results.

Yeast Two-hybrid System—The Matchmaker Gold yeast two-hybrid system was used (Clontech). In brief, full-length rat Hax-1 v1 was cloned into the pGBKTK7 vector and served as bait. The rat Hax-1 v1 transcript was split into eight different regions, on the basis of predicted domain structure, that were cloned into the pGADT7 vector and used as prey. The Gold-competent cells, provided by the manufacturer, were simultaneously transformed with the indicated bait and prey constructs and plated on the appropriate selective media. Positive interactants were then streaked on high-stringency plates for confirmation and evaluation of their relative strength, as described by the manufacturer.

Measurement of Intracellular Reactive Oxygen Species (ROS) —The OxiSelect intracellular ROS assay kit (Cell Biolabs, San Diego, CA) was used according to the protocol of the manufacturer. Cells overexpressing select Hax-1 constructs were treated with 0.33 mM H2O2, and intracellular ROS levels were measured after 1 and 4 h of treatment.

Measurement of Cytochrome c Release—The cytochrome c releasing apoptosis assay kit (Abcam, catalog no. ab65311) was used according to the instructions of the manufacturer. HEK293 cells were transfected with select Hax-1 constructs as described above and treated with 0.33 mM H2O2. A pilot time course was performed to determine the time point of maximum cytochrome c release after treatment, which was shown to be 2 h.

RESULTS

Expression Profile of Select Hax-1 Transcripts in the Rat Heart Prior to and Post-induction of Myocardial Infarction—Hax-1 v1 is expressed ubiquitously, albeit at different amounts among different tissues (10). Consistent with this, high transcript levels of Hax-1 v1 have been reported in the mammalian heart (4), whereas no such information is available for other Hax-1 variants. Using quantitative RT-PCR and variant-specific primers, we compared the expression levels of Hax-1 v1, v2, v4, and v6 transcripts in healthy and stressed rat hearts (Fig. 1A; no variant-specific primers can be designed for v3, v5, and v7). All four HAX-1 transcript variants were readily expressed in healthy rat myocardium (Fig. 1B, black bars). Interestingly, a significant increase of ~12-fold was detected in the transcript levels of v2 12 weeks after transaortic ligation and induction of myocardial infarction (Fig. 1B, gray bars). No statistically significant changes in the transcript levels of v1, v4, and v6 were observed in the stressed rat heart (Fig. 1B). The up-regulation of v2 transcripts was corroborated by a ~1.5-fold increase at the protein level in the treated hearts, compared with sham-operated control hearts, whereas the protein levels of Hax-1 v1 remained unchanged (Fig. 1, C and D).

Hax-1 v1 and v2 Have Antagonistic Roles in Regulating H2O2-induced Apoptosis—Given the increased expression levels of Hax-1 v2 in the rat heart following myocardial infarction, we set forth to examine whether and how v2 regulates cell death. We used HEK293 epithelial cells, which have been used extensively and reliably for such studies (24). We transiently transfected HEK293 cells with rat Hax-1 v1, v2, v1, and v2 or control empty vector(s) (Fig. 2A) and subsequently subjected them to different apoptotic stimuli. Notably, we routinely observed >80% efficiency for single transfections and >70% efficiency for cotransfections. We used a variety of apoptotic stimuli, including thapsigargin, which raises cytosolic Ca2+ concentration by blocking the activity of SERCA (25); calcimycin, a divalent cation ionophore that allows Ca2+ to cross membranes (26); serum starvation (27); and H2O2, which induces oxidative stress and causes cytochrome c release from the mitochondria, leading to caspase activation (28). We first measured metabolic activity using the XTT assay, which is indicative of cell viability (Fig. 2, B–F). Overexpression of Hax-1 v1 or v2 had no effect on cell viability, compared with control cells expressing the empty vector, following treatment with thapsigargin (Fig. 2B) or calcimycin (C) and after serum starvation (D). These findings are surprising given the direct association of Hax-1 v1 with major Ca2+ regulatory proteins (14, 16, 29). However, overexpression of Hax-1 v1 protected HEK293 cells from H2O2-induced death by ~1.5-fold, whereas overexpression of Hax-1 v2 exacerbated cell death by ~0.75-fold compared with control cells (Fig. 2, E and F). Importantly, cotransfection of Hax-1 v1 and v2 in HEK293 cells that yielded equivalent amounts of exogenous v1 and v2 proteins (Fig. 2A) abrogated their anti-apoptotic and prodeath effects, respectively, following exposure to H2O2 (F).

To further confirm these findings, we performed similar experiments using the human homologues of rat v1 and v2; i.e. v1 and v4, respectively (Fig. 2A). Notably, rat v1 and human v1 share ~84% sequence identity, whereas rat v2 and human v4
share ~75% identity (4). Similar to the rat variants, overexpression of human v1 in HEK293 cells increased viability by ~1.7-fold following H$_2$O$_2$ treatment, whereas overexpression of human v4 promoted cell death by ~0.7-fold (Fig. 2F). Moreover, coexpression of human v1 and v4 counteracted their individual effects on cell survival (Fig. 2F).

We then used FACS analysis to examine how ectopic expression of the rat Hax-1 variants 1 and 2 and human Hax-1 variants...
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1 and 4 affected apoptosis of HEK293 cells following exposure to H$_2$O$_2$. We performed measurements of annexin V PE and 7-amino-actinomycin D at 4 and 24 h post-treatment to capture early and late cell responses. Approximately 90% of HEK293 cells expressing rat v1, human v1, or a control empty vector were alive at 4 h following H$_2$O$_2$ treatment (Fig. 3A). Interestingly, only ~80% of HEK293 cells expressing either rat v2 or human v4 were alive at the same time point (Fig. 3A). We then measured the levels of annexin V PE and 7ADD early and late cell responses. Approximately 90% of HEK293 cells expressing rat v2 or human v4 were alive at 24 h, indicating the prodeath effects of rat v2 and human v4 (Fig. 3B). Similar measurements in HEK293 cells coexpressing equivalent amounts of rat v1 and v2 or human v1 and v4 (Fig. 2A) demonstrated that ~90% of cells were alive at 4 h, whereas 50% of cells remained alive at 24 h, similar to control cells expressing empty vectors at the same time points (Fig. 3, A and B). Taken together, these results demonstrate that rat and human v1 promote cell survival under specific stress conditions (i.e. oxidative stress) by diminishing apoptosis, whereas rat v2 and human v4 exacerbate cell death under the same stress conditions by increasing apoptosis. Interestingly, coexpression of anti-apoptotic v1 and prodeath v2/v4 counteracts their individual effects, resulting in apoptotic levels similar to those of control cells.

The Hax-1 Variants Can Form Homotypic and Heterotypic Dimers with High Affinity—To study the molecular mechanism through which Hax-1 v1 and v2/v4 counteract the effect of each other in regulating cell apoptosis, we examined their ability to form homotypic and heterotypic dimers, a property described previously for other members of the Bcl2 family (30, 31). We generated full-length recombinant rat Hax-1 v1 and v2 as GST- and His-tagged proteins (Fig. 4A) and performed a pulldown assay (B). Notably, both v1 and v2 were able to specifically and efficiently form homo- and heterodimers. To quantitate the binding affinity of these homo- and heterotypic interactions in real time, we performed surface plasmon resonance using a Biacore 3000 instrument (Fig. 4, C and D). v1 formed homodimers with a $K_d$ of ~4 nM and heterodimers with v2 with a $K_d$ of ~97 nM. Although the binding affinity of the v1 homodimer is ~25-fold higher than the binding affinity of the v1/v2 heterodimer, both interactions are strong and, most likely, of physiological significance.

FIGURE 2. Effects of Hax-1 variants on cell viability following exposure to different insults. A, immunoblot analyses of lysates collected from HEK293 cells 24 h post-transfection overexpressing rat Hax-1 v1 (RV1), v2 (RV2), RV1 and RV2, human v1 (HV1), v4 (HV4), or HV1 and HV4 and probed with a Hax-1 antibody (ab). Immunoreactive bands are denoted with colored dots for ease of identification. Red, endogenous human v1; green, exogenous rat v1; orange, exogenous rat v2; blue, endogenous and exogenous human v1, which comigrate; yellow, exogenous human v4. It is of interest to note that HEK293 cells do not express detectable levels of endogenous Hax-1 v4 under normal conditions and that endogenous and exogenous HV1 migrate slightly faster than exogenous RV1 in our gel system. To ensure equal loading, a replica immunoblot analysis was probed with an antibody to tubulin. B–E, HEK293 cells overexpressing rat Hax-1 v1 or v2 were treated with 2 µM thapsigargin for 24 h (B) or 4 µM calcimycin for 24 h (C) or were serum-starved (−FBS, lacking FBS) for 24 h (D) or exposed to 0.33 mM H$_2$O$_2$ for 4 h (E). Cell viability was measured using the XTT assay. Rat Hax-1 v1 protected H$_2$O$_2$-treated cells from cell death, as evidenced by the ~1.4-fold increase in cell viability compared with control cells (n = 3; Student’s t test; *p < 0.001; error bars show mean ± S.E.) but failed to protect thapsigargin- and calcimycin-treated or serum-starved cells. Conversely, rat Hax-1 v2 decreased cell viability of H$_2$O$_2$-treated cells by ~0.8-fold compared with control cells (n = 3; Student’s t test; *p < 0.04; error bars show mean ± S.E.) but not of thapsigargin- and calcimycin-treated or serum-starved cells. F, HEK293 cells overexpressing RV1, RV2, or RV1 and RV2 and HV1, HV4, or HV1 and HV4 were treated with 0.33 mM H$_2$O$_2$ for 4 h as in D, followed by evaluation of cell viability using the XTT assay. Similarly to the rat variants, human v1 and v4 decreased and exacerbated cell death of H$_2$O$_2$-treated cells by 20 and 10%, respectively, compared with control cells. Importantly, coexpression of rat v1 and v2 or human v1 and v4 counteracted the ant apoptotic effect of v1 and the prodeath effect of v2/v4, resulting in cell viability levels similar to those observed in control cells expressing empty vectors (n = 3; Student’s t test; **p < 0.01; ***p < 0.003; error bars show mean ± S.E.).
We were unable to efficiently capture Hax-1 v2 to CM5 or nitrilotriacetic acid chips as GST or His fusion proteins, respectively. Therefore, kinetic measurements for the v2 homodimer were not feasible.

The COOH Terminus of Hax-1 Supports Its Ability to Homo- and Heterodimerize—To determine the minimal region that supports the formation of Hax-1 homo- and heterodimers, we generated a series of deletion constructs and tested their ability to interact in the yeast two-hybrid system. We used full-length rat Hax-1 v1 as bait and three consecutive deletion constructs, referred to as A-C, as prey, containing amino acids 1–43, 35–102, and 97–278, respectively, and spanning the entire length of v1 (Fig. 5A). We also generated prey construct D, containing amino acid residues 79–106, which are unique to v7. Prey construct C, including amino acids 97–278, interacted specifically and efficiently with full-length v1 under high-stringency conditions in the yeast system (Fig. 5B). Further deletion analysis of fragment C (aa 97–278) and generation of constructs C1 (aa 97–193) and C2 (aa 189–278) demonstrated that both C1 and C2 were able to support binding to full-length v1. However, C1 did so more efficiently and to the same extent as construct C. Additional deletion analysis and generation of constructs C3 (aa 97–148), C4 (aa 127–202), C5 (aa 188–224), and C6 (aa 225–278) resulted in complete abolishment of binding to full-length v1 (Fig. 5B).

We then tested the ability of constructs C (aa 97–278), C1 (aa 97–193), and C2 (aa 189–278) to directly bind to full-length Hax-1 v1 in a pulldown assay. Therefore, we generated recombinant GST–Hax-1 v1, which was immobilized to glutathione matrices and allowed to incubate with equivalent amounts of His-tagged C, C1, or C2 proteins (Fig. 5C). We observed that His-C, His-C1, and His-C2 proteins were able to specifically and directly bind to GST–Hax-1 v1 but not control GST protein (Fig. 5D). Similarly to the yeast two-hybrid data, we consistently observed that His-C and His-C1 were retained more efficiently by GST–Hax-1 v1 compared with His-C2. Thus, it appears that the C1 region containing residues 97–193 is the minimal site that mediates the ability of Hax-1 to form homotypic and heterotypic dimers, whereas the C2 region containing amino acids 189–278 may further contribute to the homo- or heterodimerization capability of Hax-1.

Notably, the COOH-terminal region of Hax-1 (aa 97–278), which also contains the homo- and heterodimerization site, as our experiments indicated, is shared by all known Hax-1 variants and supports binding to a number of other proteins, as reported previously, including phospholamban, SERCA2, HtrA2, PARL, caspase 9, and HS1 (4).

Overexpression of Antiapoptotic or Prodeath Hax-1 Variants Fails to Modulate the Levels of Intracellular ROS but Alters the Cytoplasmic Levels of Cytochrome c following H2O2 Treatment—We then investigated whether the antiapoptotic effects of rat and human v1 and the prodeath effects of rat v2 and human v4 are due to their ability to regulate intracellular ROS levels. HEK293 cells overexpressing rat Hax-1 v1, v2, or v1 and v2 and human Hax-1 v1, v4, or v1 and v4 were treated with H2O2, and the levels of intracellular ROS were measured 1 and 4 h post-treatment. As expected, all cell populations exposed to H2O2 exhibited significantly higher levels of intracellular ROS compared with untreated HEK293 cells expressing empty vector(s) (Fig. 6A). Interestingly though, we did not observe statistically significant differences in the levels of intracellular ROS at 1 h (Fig. 6A) or 4 h (not shown) following H2O2 treatment among cell groups overexpressing v1 or v2/v4 or cell groups coexpressing v1 and v2/v4 compared with control cells containing empty vector(s). Therefore, we then examined whether the opposing effects of v1 and v2/v4 are exerted via regulation of the levels of cytosolic cytochrome c, which we measured in the aforementioned HEK293 cell groups (Fig. 6B). We initially performed a
time course (0.5–24 h, data not shown) to determine the time point of maximal cytochrome c release, which we found to be 2 h post-treatment. HEK293 cells overexpressing rat v2 or human v4 exhibited significantly higher levels of cytosolic cytochrome c (Fig. 6B, 1.4-fold increase) compared with cells overexpressing rat v1, rat v1 and v2, human v1 and v4, or empty vector(s), as evidenced by densitometry of the respective immunoreactive bands. Taken together, these findings indicate that the antagonistic roles of v1 and v2/v4 are not exerted via modulation of intracellular ROS levels but through regulation of cytochrome c release.

**DISCUSSION**

Hax-1 v1 is a ubiquitously expressed protein with established antiapoptotic activity that has been studied extensively in humans and rodents (4, 19). Contrary to the mouse gene, the human and rat HAX-1 genes are heavily spliced, giving rise to at least seven distinct variants that primarily differ in the NH2 terminus (1). In this study, we examined the expression profile of Hax-1 variants in normal and stressed rat myocardia. We observed a significant increase in the transcript and protein levels of Hax-1 v2 12 weeks post-induction of myocardial infarction. Consistent with this, rat v2 and its human homologue v4 exacerbated cell death under select stress conditions that led to oxidative stress and abrogated the antiapoptotic effect of rat and human v1, respectively. Rat v2 and human v4 are structurally similar to the rat and human v1. They only differ in the retention of exon 2, which is present in all known v1 proteins but is partly spliced out in the rat v2 and human v4 isoforms (1). The spliced-out portion of exon 2 encodes 61 amino acids in rats and 48 amino acids in humans, comprising the predicted BH1 and BH2 domains and flanking sequence. Therefore, it appears that the BH1 and BH2 domains present in Hax-1 v1 confer their antiapoptotic activity, as reported previously for antiapoptotic members of the BCL2 family (32–34). Notably, although the existence of the BH1 and BH2 domains in Hax-1 is controversial, our findings highlight the functional importance of this region of Hax-1 encoded by exon 2.
Previous studies have demonstrated that Hax-1 v1 can interact specifically and directly with several proteins that are intimately involved in the regulation of apoptosis via distinct mechanisms. These include phospholamban (9) and SERCA (14), which are major modulators of Ca^{2+} cycling; mitochondrial proteases presenilins-associated rhomboid-like protein (PARL); high temperature-regulated A2 (HtrA2) (18); and cytoplasmic procaspase 9 (5, 18). Consistent with these findings, it has been postulated that direct binding of Hax-1 v1 to phospholamban and SERCA modulates Ca^{2+} homeostasis (14, 17, 35). Alternatively, direct binding of Hax-1 v1 to HtrA2 and PARL facilitates the PARL-mediated activation of HtrA2 (18). Activated HtrA2 prevents the accumulation of proapoptotic BAX and, thus, antagonizes the anti-apoptotic activity of v1. Therefore, it is possible that Hax-1 v2/v4 may directly bind to HtrA2 and PARL, leading to their inactivation. Conversely, Hax-1 homodimers or heterodimers may act independently of other Hax-1 binding partners. Thus, homodimerization may be required for v1 to exert its antiapoptotic effect, and for v2/v4 to potentiate cell death following insult. Alternatively, heterodimerization may neutralize the individual effects of v1 or v2/v4 via sequestration. Thus, overexpression of rat v2 or human v4 may promote cytochrome c release following H_{2}O_{2} treatment by heterodimerizing and sequestering endogenous v1, thus preventing it from facilitating PARL-mediated activation of HtrA2 and BAX removal from the outer mitochondrial membrane (18). Therefore, it becomes apparent that there is a high degree of previously unforeseen complexity that characterizes the homotypic, heterotypic, and allotopic binding interactions of the Hax-1 proteins, which may depend on their ratio and subcellular localization as well as the presence and type of stress exerted to the cell.

The antagonistic roles of Hax-1 v1 and v2/v4 are reminiscent of the opposing roles of the BCL-2-X splice variants, BCL-Xl and BCL-Xs (37, 38). BCL-Xl binds to and sequesters BAX and BAK (BCL2 homologous antagonist/killer), inhibiting them from...
These results indicate that Hax-1 v1 and v2/v4 do not regulate H$_2$O$_2$-induced apoptosis, whereas treated cells expressing the different Hax-1 variants or combinations thereof showed mean ± S.E. (n = 3; Student’s t test; *, p < 0.03; error bars show mean ± S.E.). However, there was no statistically significant difference in intracellular ROS levels between treated cells expressing empty vector(s) and treated cells expressing the different Hax-1 variants or combinations thereof (n = 3; Student’s t test; *, p > 0.03; error bars show mean ± S.E.). These results indicate that Hax-1 v1 and v2/v4 do not regulate H$_2$O$_2$-induced apoptosis by modulation of intracellular ROS levels. B, HEK293 cells overexpressing RV1, RV2, and V1 and RV2 or RV1 and HV1 and HV4 or empty vector(s) were treated with 0.33 mM H$_2$O$_2$ for 2 h. Cell groups were then subjected to subcellular fractionation to collect the cytoplasmic fractions, using the Abcam cytochrome c release kit, which were analyzed by immunoblotting using antibodies (ab) to cytochrome c. As expected, untreated cells contain undetectable levels of cytoplasmic cytochrome c, whereas treated cells expressing empty vector(s), RV1, HV1, RV1 and RV2 or RV1 and HV1 and HV4 have similar, yet low, amounts of cytoplasmic cytochrome c. In contrast, treated cells expressing RV2 or HV4 show notably higher levels of cytosolic cytochrome c (~1.4-fold increase compared with control cells), indicating its release from the mitochondria and the initiation of apoptosis. GAPDH served as a loading control.

forming pores in the outer mitochondrial membrane and, thus, preventing cytochrome c release (33, 37). BCL-Xs, similarly to Hax-1 v2/v4, lacks the BH1 and BH2 domains present in BCL-XI and Hax-1 v1 and promotes cell death in the presence of a stress stimulus (39). Although the exact mechanism of action of BCL-Xs is still unknown, it has been shown that it can bind to and inhibit or sequester antiapoptotic proteins BCL-XI and BCL2 (32, 38, 40—42). Notably, the proapoptotic effect of BCL-Xs is counteracted by overexpression of BCL2 or BCL-XI (30), similarly to Hax-1 v2/v4 and v1.

Several antiapoptotic BCL2 family members, such as BCL2 and BCL-XI, have been shown to prevent the accumulation of ROS within cells, thus protecting against ROS-induced apoptosis (43, 44). Interestingly, cells overexpressing Hax-1 v1 failed to reduce the levels of intracellular ROS following H$_2$O$_2$ treat-

ment, compared with control cells, but exhibited reduced levels of cytoplasmic cytochrome c. Conversely, cells overexpressing Hax-1 v2/v4 failed to increase the levels of intracellular ROS but displayed increased levels of cytoplasmic cytochrome c. These findings indicate that the antiapoptotic and prodeath effects of Hax-1 proteins are not mediated via regulation of intracellular ROS levels but through modulation of cytochrome c release, possibly via the HtrA2/PARL pathway (18, 45).

Taken together, our findings demonstrate that Hax-1 comprises a family of apoptotic regulators with antagonistic roles in response to oxidative stress. The ability of Hax-1 proteins to form homo- and heterodimers and to interact with diverse proteins involved in the regulation of cell fate indicates that modulation of cell survival or death via the Hax-1 family is highly complex and manifold. Further work is required to delineate the precise molecular mechanisms via which Hax-1 proteins may promote cell survival or apoptosis and to establish how their ratio; subcellular distribution; and preferential homotypic, heterotypic, or allotypic binding interactions contribute to the determination of cell fate under stress conditions.

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