Hax1 lacks BH modules and is peripherally associated to heavy membranes: implications for Omi/HtrA2 and PARL activity in the regulation of mitochondrial stress and apoptosis

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Hax1 has an important role in immunodeficiency syndromes and apoptosis. A recent report (Chao et al., Nature, 2008) proposed that the Bcl-2-family-related protein, Hax1, suppresses apoptosis in lymphocytes and neurons through a mechanism that involves its association to the inner mitochondrial membrane rhomboid protease PARL, to proteolytically activate the serine protease Omi/HtrA2 and eliminate active Bax. This model implies that the control of cell-type sensitivity to pro-apoptotic stimuli is governed by the PARL/Hax1 complex in the mitochondria intermembrane space and, more generally, that Bcl-2-family-related proteins can control mitochondrial outer-membrane permeabilization from inside the mitochondrion. Further, it defines a novel, anti-apoptotic Opa1-independent pathway for PARL. In this study, we present evidence that, in vivo, the activity of Hax1 cannot be mechanistically coupled to PARL because the two proteins are confined in distinct cellular compartments and their interaction in vitro is an artifact. We also show by sequence analysis and secondary structure prediction that Hax1 is extremely unlikely to be a Bcl-2-family-related protein because it lacks Bcl-2 homology modules. These results indicate a different function and mechanism of Hax1 in apoptosis and re-opens the question of whether mammalian PARL, in addition to apoptosis, regulates mitochondrial stress response through Omi/HtrA2 processing.
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Mitochondria are key players in central cellular processes, such as ATP production, Ca^{2+} signaling and apoptosis. Mitochondrial involvement in apoptosis has been thoroughly documented during the last decade. Its two main features include the release of proteins from the mitochondrial intermembrane space (IMS) and the initiation of a program of dysfunction that includes the loss of the proton electro-chemical gradient across the inner mitochondrial membrane (IMM).[1] These two cascades of events seem to be mediated by the crosstalk of several molecular mechanisms that are still not fully characterized.[2] Nevertheless, the general consensus is that during apoptosis, mitochondria release cytochrome c and other proteins that cooperate to execute programmed cell death.[3–5]

The function of mitochondria in the regulation and amplification of the apoptotic cascade is regulated by members of the Bcl-2 protein family.[6,7] These are cytosolic proteins that, under steady state conditions, are mainly peripherally associated to heavy membranes,[8] and that share a limited structural similarity with Bcl-2 in the so called Bcl-2 homology (BH) module. Bcl-2-family-related proteins participate in the same process of regulation of apoptosis and are classified as pro- and anti-apoptotic, depending on their effect on programmed cell death. Pro-apoptotic members are further subdivided into 'multidomain' ones that share the BH1, BH2 and BH3 modules, and the 'BH3-only' proteins that only share the BH3 module. The pro-apoptotic BH3-only proteins 'sense' the death stimuli and transduce them to mitochondria, where they activate the 'multidomain' pro-apoptotic proteins Bax and Bak, ultimately resulting in mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. With respect to the anti-apoptotic Bcl-2 family members, two models of activity have been proposed and are the subject of intense investigations. In the first model, these proteins are endogenous inhibitors of the multidomain pro-apoptotic proteins, requiring to be antagonized by 'BH3-only' molecules; in the second model, the anti-apoptotic Bcl-2 family proteins act by sequestering BH3-only proteins, a possibility strongly supported by recent, elegant studies.[9] However, a recent report puzzled these two models by proposing that Hax1, a purported Bcl-2-family-related protein of the mitochondrial IMS, mediates the elimination of active Bax, thereby introducing the concept that anti-apoptotic Bcl-2 proteins can antagonize MOMP also from inside the mitochondria through the activation of a proteolytic cascade.[10]

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To ensure the complete release of cytochrome c, the architecture of the mitochondrial reticulum and the ultrastructure of the organelle changes in the early stages of apoptosis.[11–14] A combination of electron tomography and physiological measurements identified a pathway of cristae remodeling characterized by the widening of the narrow tubular junction and by the fusion of individual cristae. These morphological changes support the mobilization of cytochrome c from the cristae to the IMS, and eventually to the cytosol.[15] Mechanistically, the mitochondrial rhomboid protease PARL and the dynamin-related GTPase Opa1, two proteins of the IMM, participate in the control of the shape and structure of the cristae and of the cristae junctions.[16,17] Under steady state conditions, active Opa1 can prevent the widening of the cristae junctions by forming an oligomer that functions as a molecular staple between the adjacent membranes of the cristae. This high molecular weight complex contains both an IMM-bound form of Opa1 and an IMS-soluble one. Generation of the latter form requires the rhomboid protease PARL, whose ablation is lethal in adult mice owing to a pathology caused by excessive apoptosis in multiple tissues.[16,17] A similar phenotype was recently observed in mice lacking Hax1, which also displayed reduced levels of a cleaved form of Omi/HtrA2,[10] a serine protease of the IMS implicated in oxidative stress and apoptosis.[18] These observations and the finding that Hax1 could be co-immunoprecipitated with PARL suggested a model in which Hax1 presents Omi/HtrA2 to PARL, to generate a form of Omi/HtrA2 that may proteolytically antagonize from the IMS active Bax during MOMP,[10] thereby defining a novel anti-apoptotic Opa1-independent pathway for PARL.[16,17,19]

Our study shows that Hax1 is not a *bona fide* Bcl-2-family-related protein because it does not share any sequence similarity with BH modules, and its confidently predicted secondary structure is incompatible with the presence of BH modules. We further show that, *in vivo*, the activity of Hax1 cannot be mechanistically coupled to PARL because the two proteins are confined in different cellular compartments, and their interaction *in vitro* is an artifact. Hax1 has a key role in autosomal recessive severe congenital neutropenia,[20] a primary immunodeficiency syndrome associated with increased apoptosis in myeloid cells; therefore, correcting the mechanism of Hax1 activity remains an outstanding question, which has to be addressed to decipher the molecular pathways that link mitochondrial stress response to apoptosis.

Results

Hax1 lacks BH modules. Hax1 was initially proposed to be a Bcl-2 family protein on the basis of purported structural similarities to Bcl-2 family members, including the presence of BH1- and BH2-like modules and a C-terminal transmembrane domain.[21] Recent studies have embraced this notion,[10] contributing to consolidate Hax1 as a member of this important family of proteins. However, our sequence analysis and structure prediction do not support the presence of any BH modules in Hax1. Indeed, the purported BH1 and BH2 modules, located at position 37–56 and 74–89,

respectively, are not recognized by conserved protein domain search, even with the most relaxed threshold. Further, multiple secondary structure predictions show with full consistency that the regions of Hax1, which were previously aligned with the BH1 and BH2 modules, are largely disordered, whereas the *bona fide* BH1 and BH2 modules are stable hairpins formed by hydrophobic α-helices.[22,23] In addition, the corresponding regions of the Hax1 sequence are not well conserved even in closely related animals, such as mammals, and show no sequence conservation at all in more distantly related species (Figure 1), which would be incompatible with the key roles of these regions in the function of Hax1 in apoptosis as a Bcl-2 protein. Our analysis shows instead that Hax1 is an α/β-protein that contains a strongly predicted and relatively well-conserved, in animals, three-strand β-sheet near the C-terminus (Figure 1), a structural element absent in Bcl-2 proteins. Interestingly, Hax1 also contains a conspicuous pattern of three universally conserved aspartates embedded in a predicted disordered loop, which is suggestive of functionally important metal (possibly, calcium)-binding residues. None of these structural elements are present in any of the Bcl-2 proteins. We conclude that it is exceedingly unlikely that Hax1 is a member of the Bcl-2 family.

Hax1 is not an integral membrane protein. At the C-terminus of Hax1, there is a strongly predicted and conserved C-terminal α-helix, which has been purported to constitute the transmembrane domain that anchors Hax1 to both mitochondrial membranes.[10] Multiple methods of transmembrane region prediction, as well as visual inspection of Hax1 sequence for long hydrophobic stretches, indicate that Hax1 does not contain such transmembrane domain, either near the C-terminus or anywhere within the protein sequence (Figure 1). To span the lipid bilayer, an α-helix must be composed by a minimum of about 20 amino acids, mainly hydrophobic.[24] The purported transmembrane domain of Hax1 consists in an α-helix of 16 amino acids, 4 of which are charged and 1 is polar (RPPALDDAFSILDLFL), which could not form a transmembrane domain capable of anchoring Hax1 within a lipid bilayer. This analysis is consistent with our experimental data from alkaline and high salt extraction of heavy membranes prepared from HeLa cells, which showed that endogenous Hax1 is peripherally associated, but not integrated, to these membranes (Figure 2). We conclude that Hax1 is not an integral membrane protein and suggest that the conserved C-terminal domain might be functionally important in coordinating Hax1 interaction with other proteins.

Hax1 is not localized inside the mitochondria. A recent report claimed that endogenous Hax1 is localized on the inner and on the outer mitochondrial membrane, where it is exposed to the IMS.[10] However, such dual membrane localization and protein topology are at odd with those of every other known *bona fide* mitochondrial protein, as well as with the known mechanisms of protein import in the organelle. Hax1 does not contain cysteine residues required for IMS protein import through the MIA pathway,[25] or any predictable

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SS_PRPROT        cchh hhhhcc     cccccc     cccccccccccccccccccc     ccccccccccccccccccccccccccccccccccccccc
SS_JPRED         cchh hhhhcc     cccccc     cccccccccccccccccccc     ccccccccccccccccccccccccccccccccccccccc
SS_PSIPRED       cchh hhhhhh     cccccc     cccccccccccccccccccc     ccccccccccccccccccccccccccccccccccccccc
SS_BH1                                        hhhhhhhhhccccchhhhhhhhhhhhhhhh
                                            +++++++++"BH1"+++++++
HAX1_HUMAN       MSLF-DLFRGFF    GFPGPR     -SHRDPFFGGMTRDEDDD-E---EEEEEGGSWGRGNPRFHSPQHPPEEFGFGFSFSPGG---
HAX1_DOG         MSFF-DLFRGFF    GLSGPR     -SHRDPFFGGMTRDEDEDDE---EEEEEAAPWSHGSSRSEGPQ-TPEEFGFGFTFTPGG---
HAX1_MOUSE       MSVF-DLFRGFF    GFPGPR     -SHRDPFFGGMTRDDDDDDDDDDEAEEDRGAWGRESYAFDGSQ-PPEEF--GFSFSPRG---
HAX1_PLATIPUS    GSAL-TGRRKRM 21 GDGGTY     -RRRDPFFGGMTREDDDEDE---EEEEEGG------GRPWAPRPPEEEFTFRFRFGPGGEGG
HAX1_FROG        MSLF-ELFRRFF    E-PGGR     ---DPFFGGMTQDDDDDDDDDDEEEGE-NFGYPFA-----RPPGSH--FGFSFSGPGRD--
HAX1_DANIO       MSVF-DLFRGFF    GVPGGH  3  DGRRDPFFDGMIHEDDDDE----DEDDFNRPHR---------DPFDDAFRFGFSFGPGG---
HAX1_LANCELET    MSLH-DLFRGLF    GFHGGH     RQPPPEYFDENED---LDDDDDDEHEED-SFGHDFGA-----------FGFRGGPGFE--
HAX1_WASP        MPFF-EFFRNLF    GK-GPA 11  QRYRDGFRNPIWQ-----TDDDEDDISDF-SNRHPANRF-----------QFRIFSD----
HAX1_SEA_URCHIN  MSRFDDIFDSFF    GR-SGF 28  SSPGSRFYSEQPH-----DMDDDDNYEGP-PGRQGPFPG-----------GGAIFS-----

SS_PRPROT        ccc         cccc  ccccchhhhhhhhhhcccccccc   cccccccccccc cc   ccccccccc    ccccccccccc  cccc
SS_JPRED         cee         cccc  chhhhhhhhhhhhhhcccccccc   cccccccccccc cc   ccccccccc    chhhhhhhcc   cccc
SS_PSIPRED       ccc         cccc  cchhhhhhhhhhhhhhcccccccc  cccccccccccc cc   ccccccccc    cccchhhhcc   cccc
SS_BH2             hhh           hhhh  ccccchhhh
                   +++        "BH2"   +++++++++
HAX1_HUMAN       GIRF-------HDNF--GFDDLVRDFNSIFSDMGAWTLPS--HPPELPGP--E-SE----TPGERLREG---QTIRDSMLKY--PDSH
HAX1_DOG         -MRF-------HDNF--GFDDLIRDFNNIFSEMEAWTLPS--RPSELPGPGPE-SE----TPGERREG---QTIRDSMLKY--PDSH
HAX1_MOUSE       GMRF-------HGNF--GFDDLVRDFNSIFSEMGAWTLPS--HSPELPGP--E-SE----TPGERLREG---QTIRDSMLKY--PDSH
HAX1_PLATIPUS    GLHF-------HDNF--GFDELVRDFNRIFSQMDTWTLPA--RRPELPGA--E-AEPG--PPGPSGSPGGRRETIRDSMLKY--PDSQ
HAX1_FROG        --PM-------ANVF--GFEEFFRDFNELFADFGSITQ----VVPELPGVDPP-------LQRPEVPRG---KSIRDFMLKS--PDSH
HAX1_DANIO       -ARF-------EEPQ--MFGQIFRDMEEMFAGLGRFDERHGFGPRGFPSIEAPPPQEGVEKGRSGTGSG---NPIRDFMLKS--PDR-
HAX1_LANCELET    --EFGGDDFGMSDMMK-HFDEMFHTFDELFRQLGTVE----FPPLSPPHRP---GVPGMEPPSSGPGHE---RSLRDRMLKE--PGA-
HAX1_WASP        -------PFEMTRFFETQMDDMMRNFFGFGNGFGNDT--NIFLPFGNENALPM---------PGENPVG---KGPRDEVLKAEVPDS-
HAX1_SEA_URCHIN  -------------FNNEMNDFFKLFDDMFKSFGIAD----FPPLDVPRISPS---------SPAQPEA---KAPRDEMLKE--PDS-

SS_PRPROT          cccc  ccccccc   cccccccccccccccccccccc cccc   cccccccccccccccccccc ccccccccc    ccccc
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HAX1_LANCELET    IPEGGSSRSEQPKSEQPIPKRYPQ-FEDFW-KNPFGQLPPSPWEKP---PSSPQDKVDKDLDAQISA--SDLDAVLP  7  PQGRS
HAX1_WASP        -------KLGLDDFISGLP--FSN-RKFGG-KGPVDEVLKPSYEMP----DSNSKKIDSDIDGKIKS--DELAKIWK 16  FSIRS
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SS_PSIPRED       ccceeeeeeeeeecccccceeeeeeeeecccccceeee eeec   cccc   ccccccccc    ccccc  cchhhhhhhhhhhhhcccc
                                                                                   +++++   "TMD"++++++
HAX1_HUMAN       YFKSISVTKITKPDGIVEERRTVVDSEGRTETT-VTRHE--ADSS----PRGDPESP    RPPAL--DDAFSILDLFLGRWFRSR
HAX1_DOG         YFKSVTVTKITKPDGTVEERRTVVDSEGRTETT-VTHQE--ADGS----PRDDPESP    TPPAL--DDAYSILDLFLGRWFRSR
HAX1_MOUSE       YFKSISVTKITKPDGTVEERRTVVDSEGRTETT-VTHQE--AHDS----SRSDPDSQ    RSSAL--DDPFSILDLFLGRWFRSR
HAX1_PLATIPUS    YYQSVSVTTVLAPDGTVEERRTVVDSEGRTETTTVTRRG--GDDA----PAG---AP 13 RTPAL--GDATSVLDLLLGRWFRPR
HAX1_FROG        FFQSVSVSKIVRPDGTIEERRTVRDGQGNSSTT-VTVQR--GDEILSSETQDGPQGP 18 SPPDL--SDSQTLLSRILQKWFSQR
HAX1_DANIO       FFKSVSVTKVVRPDGTVEERRTVRDGEGNEETT-VTISERPGGQDRPVLDQSGPLMP    GGSDM--QDDFSMFSKFF-RGFR-S
HAX1_LANCELET    FFKSITTTTIRGPDGKVEQRRTVRDGSGNEETV-VTRND--GDQTHTVVTKRDPSGR 32 GQPRLPDDNTASIFYNLFGSWFGGK
HAX1_WASP        FGSSVSTQIVRRPDGSMEERRTVRDSDGNEEIK-ITRQI--GDKMHTIITKRAKDGS 18 TPPED-NRSDGFPWHKFFGPNPK-L
HAX1_SEA_URCHIN  YSRSISIQTIRRPDGTSETRRTERDGQGNVTTT-VTTGP--DDKPSPGSTEPRRMEP 35 GRQEG-DENDDSMYKKFFGSWFK-P
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Figure 1 Hax1 does not contain BH1 or BH2 modules, or a transmembrane domain. Multiple alignment of selected Hax1 sequences from diverse animals. The numbers between aligned blocks indicate poorly conserved sequence segments that are not shown. Secondary structure (SS) predicted with three methods is shown above the alignment. c, random coil (disordered structure); h, α-helix; e, β-strand (extended conformation). Amino acid residues that are conserved in all aligned sequences are shown in bold type, and the three invariant aspartates that comprise a putative metal-binding site are shown by reverse shading. The positions of purported BH1 and BH2 modules are shown above the respective regions of the alignment using the alignment from Sharp *et al.*[21] for BH1, and arbitrarily centering the alignment on a conserved hydrophobic residue for BH2. 'SS_BH' denotes the consensus secondary structure motifs of BH1 and BH2 modules derived from multiple crystal and NMR structures of Bcl-2-family proteins.[23] The position of the transmembrane domain (TMD) predicted in Sharp *et al.*,[21] but not in our analysis, is indicated in the C-terminal block of the alignment

mitochondrial import peptide. Further, recent extensive proteomic studies do not list Hax1 in the compendium of mitochondrial proteins.[26,27] Consistent with these findings, our data show that heavy membranes contain Hax1, but that the protein is absent in highly purified, intact mouse liver mitochondria preparations (Figure 3). We conclude that

Hax1 is not imported inside the mitochondria, although it might be peripherally associated to the organelle.

PARL association to Hax1 is unspecific. PARL is a 7-transmembrane domain-containing protein of the IMM.[28,29] Topological studies have shown that the
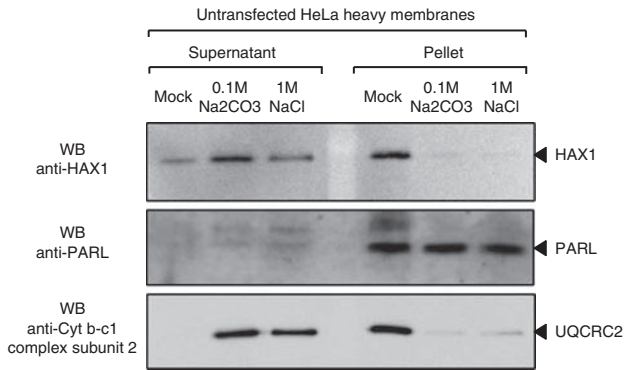
Figure 2 Hax1 is not an integral membrane protein. Alkaline extraction of heavy membranes isolated from HeLa cells (200 μg); whereas membrane-associated proteins and proteins associated to membrane-bound proteins (e.g. UQCRC2) are solubilized in the supernatant, integral membrane proteins like PARL are recovered in the membrane pellet. Lack of Hax1 integration in heavy membranes is consistent with our computational analysis (Figure 1), which does not predict any potential transmembrane domain
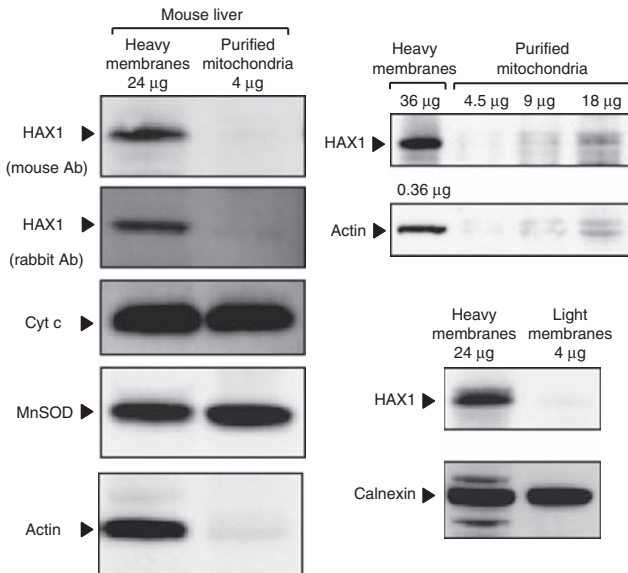

Figure 3 Hax1 is not imported in mitochondria but is peripherally associated to heavy membranes. Immunoblot analysis of Percoll-purified fractions of mouse livers. In this preparation, lack of cross-contaminating organelles was assayed by electron microscopy (not shown); inner and outer mitochondrial membrane integrity was tested for presence of diffusible proteins of the IMS (cytochrome c) and mitochondrial matrix (MnSOD); purity from membranes-associated cytosolic proteins by the absence of actin. The left panel shows that, on normalization for cytochrome c and MnSOD, endogenous Hax1 is detected in heavy membranes but not purified mitochondria, a finding consistent with lack of Hax1 in the human and murine mitochondrial proteome[26]

N-terminus of the protease is exposed to the matrix and the C-terminus to the IMS[29] (Figure 4a). PARL contains three loops exposed to the IMS: one large Loop-A that is functionally dispensable for PARL proteolytic activity (Figure 4a and b)[29] and seems to coordinate the interaction with OPA1,[17] and two very small loops that are a part of the rhomboid domain.[28,30]

Recently, co-immunoprecipitation studies were used to propose a mechanism in which Hax1 interaction with PARL allows presentation of the serine protease Omi/HtrA2 to the rhomboid protease to generate a cleaved active form of Omi/HtrA2 in the IMS.[10] As this model is incompatible with the notion that Hax1 is not targeted to the IMS (Figure 3; Pagliarini et al.[26] and Yamada et al.[27]), we investigated the unspecific nature of the reported PARL/Hax1 interaction[10] by co-transfecting HEK293 cells with constructs expressing wild-type Hax1 and mutant forms of PARL that lack mitochondrial import. Data showed that Hax1 could be co-immunoprecipitated with PARL irrespective of the fact that the latter was neither properly targeted nor folded in the IMM (data not shown), suggesting that Hax1 association is not mediated by IMS domain(s) of PARL but, rather, by unspecific hydrophobic interaction between the two misfolded proteins. To address this possibility, we deleted the only two IMS domains of PARL that could potentially bind to Hax1 and confirmed their dispensability for co-immunoprecipitating Hax1 (Figure 4b). Further, we excluded their role as *bona fide* IMS Hax1-binding domain by means of a mammalian two-hybrid system (Figure 4c).

To further validate the unspecific nature of the PARL/Hax1 association, we tested whether Hax1 co-immunoprecipitation with PARL occurs after cellular lysis by mixing (i) lysates of cells that were independently transfected with constructs expressing PARL and Hax1 (Figure 5a) and (ii) lysates of heavy membranes isolated from *Parl*−/− and *Hax1*−/− MEFs (Figure 5b). In both cases, PARL/Hax1 association could be recreated, supporting the conclusion that the interaction between these two proteins is an *in vitro* artifact that occurs post lysis through unspecific hydrophobic interactions.

PARL activity does not require Hax1. Although some types of proteases require substrate presentation by an accessory protein, rhomboids do not seem to need one.[31,32] The recently proposed Hax1/PARL model assigns a PARL substrate-presenting function to Hax1.[10,33] However, this seems to be an unlikely possibility because loss of Hax1 function only partially reduces the expression of a cleaved form of Omi/HtrA2.[10] Further, genetic ablation of *Hax1* compromises neither Opa1 cleavage[10] nor PARL cleavage (Figure 6), an N-terminal processing that requires PARL activity supplied *in trans*.[34] Together with the non-IMS localization of Hax1 (Figure 3; Pagliarini *et al.*[26] and Yamada *et al.*[27]), these evidences indicate that the protein is not a PARL substrate-presenting protein and that PARL rhomboid activity does not require Hax1.

Discussion

This study arises from a recent report that addressed the role of Hax1 in mediating the processing of the mitochondrial stress-related Omi/HtrA2 protease to allow the survival of lymphocytes and neurons.[10] In this study, cell-type sensitivity to pro-apoptotic stimuli was proposed to be governed by the formation of a complex between Hax1, a purported Bcl-2 family-related protein, and PARL, a mitochondrial rhomboid protease implicated in apoptosis and mitochondria dynamics regulation.[17,29] Mechanistically, Hax1/PARL
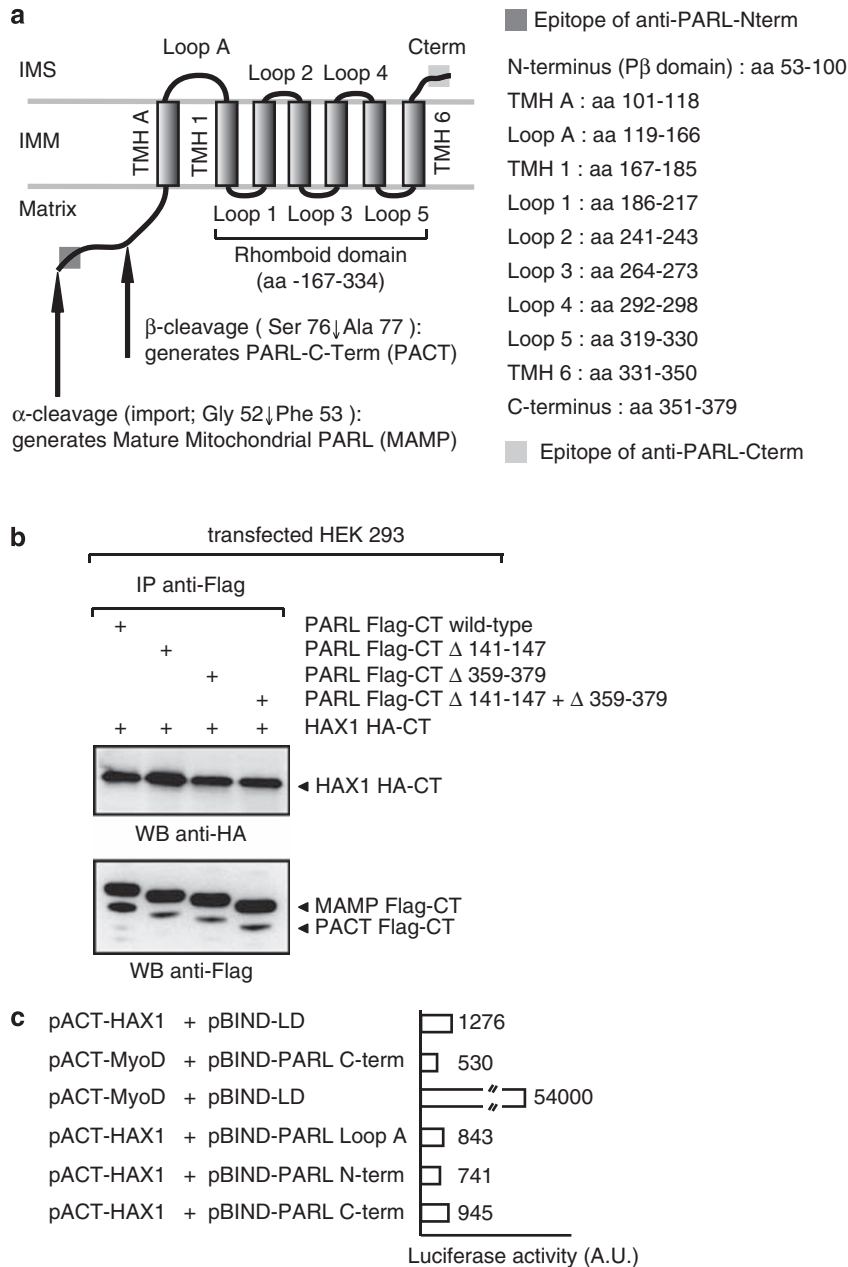
a

Epitope of anti-PARL-Nterm

N-terminus (Pβ domain) : aa 53-100

TMH A : aa 101-118

Loop A : aa 119-166

TMH 1 : aa 167-185

Loop 1 : aa 186-217

Loop 2 : aa 241-243

Loop 3 : aa 264-273

Loop 4 : aa 292-298

Loop 5 : aa 319-330

TMH 6 : aa 331-350

C-terminus : aa 351-379

Epitope of anti-PARL-Cterm

β-cleavage (Ser 76↓Ala 77): generates PARL-C-Term (PACT)

α-cleavage (import; Gly 52↓Phe 53): generates Mature Mitochondrial PARL (MAMP)

Rhomboid domain (aa -167-334)

b

Figure 4 PARL does not have a domain that coordinates interaction to Hax1 in the IMS. (**a**) Schematic representation of the topology and domain composition of PARL.[28,30] (**b**) Co-immunoprecipitation of Hax1 with mutant forms of PARL in which the IMS domains (loop-A and C-terminus) have been deleted. Note that PACT formation in indicates that the mutant proteins are correctly imported and folded in the inner membrane. (**c**) Mammalian two-hybrid assays fails to identify an IMS domain in PARL that could mediate interaction to Hax1. For a positive control reaction, pBIND-LD and pACT-MyoD control vectors are cotransfected along with pG5luc. Values, expressed as arbitrary units (A.U.), indicate mean of three independent experiments done in triplicates

association in the IMS was reported to allow the recruitment and presentation of Omi/HtrA2 to PARL,[10,33] to generate a cleaved active form of Omi/HtrA2 that, in turn, could antagonize MOMP from the IMS through proteolytic elimination of active Bax,[10] a concept that, however, is at odd with the notion that active Bax does not expose to the IMS, a cleavable domain.[6]

For Hax1 to recruit and present Omi/HtrA2 to PARL, the protein must be localized within the IMS. However, although we observed an association of Hax1 with the heavy membrane fraction, we did not detect Hax1 in Percoll-purified mouse liver mitochondria (Figure 3), a finding consistent with the absence of Hax1 from the mitochondrial proteome,[26,27] as well as with the lack of a predictable mitochondrial targeting peptide or amino acid signature in animal orthologs of this protein (Figure 1 and data not shown). The association of Hax1 with heavy membranes is peripheral, as it was removed by alkaline and high salt extraction (Figure 2). Thus, contrary to previous reports,[10,35] we found that endogenous Hax1 is neither resident inside the mitochondria nor anchored to the
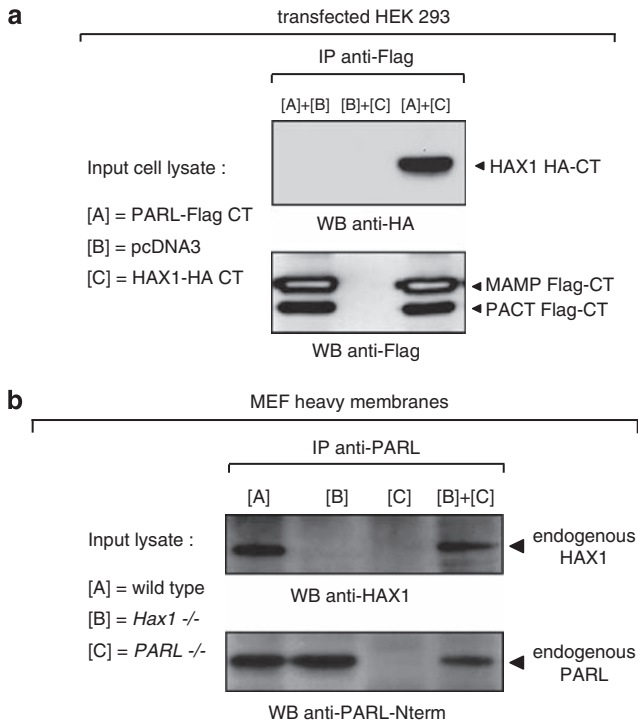
Figure 5 Hax1 binding to PARL is unspecific. (**a**) *In vitro* reconstitution of PARL/Hax1 complex by mixing lysates of HEK293 cells transfected with a construct expressing PARL-Flag-CT or Hax-HA-CT. (**b**) *In vitro* reconstitution of PARL/Hax1 complex by mixing lysates of heavy membranes (150 μg of proteins per genotype used) isolated from MEFs $+/+$, *Hax1* $-/-$ or *Parl* $-/-$

Figure 6 PARL proteolytic activity does not require Hax1. The upper panel shows that the level of expression of the mitochondrial mature form of PARL, MAMP, is not altered in any of the indicated genotypes. The middle panel indicates that genetic ablation of *Hax1* does not impair the generation of PACT, a shorter form of MAMP that requires PARL activity supplied *in trans*.[34] The lower panel shows the expression levels of Hax1 in mouse *Hax1* $+/+$, $+/-$ and $-/-$ brain lysates

membranes of the organelle. Although our findings cannot exclude the possibility that a subtle amount of Hax1 could be targeted inside the mitochondria, they nevertheless indicate that the loss of the heavy membrane-associated form of Hax1 is likely responsible for the lethal phenotype displayed by *Hax1*$-/-$ mice, as well as for the reduced levels of processed Omi/HtrA2.[10]

In this study, we tested the specificity of the interaction between Hax1 and PARL, a highly hydrophobic protein of the IMM.[28,29] Consistent with previous reports,[10] we also observed that Hax1 can be co-immunoprecipitated with PARL, but not vice versa (not shown). However, the specificity of this association is dubious at best. Mixing lysates of cells that were independently transfected with constructs expressing PARL-Flag-CT and Hax1-HA-CT reconstituted PARL/Hax1 interaction (Figure 5a); similarly, the interaction of endogenous PARL and Hax1 could be recreated by mixing lysates of heavy membranes isolated from *Parl*$-/-$ and *Hax1*$-/-$ MEFs (Figure 5b). Furthermore, we could not identify any domain within PARL that could mediate interaction to Hax1 (Figure 4). Together, these results suggest that Hax1 does not specifically bind PARL and thus it cannot present a substrate to PARL.[10,33] This conclusion is consistent with the observation that genetic ablation of *Hax1* does not fully abolish Omi/HtrA2 processing,[10] it is not required for the cleavage of OPA1[10] and it does not affect PARL β-cleavage (Figure 6), which is self-regulated.[34]

Whether mammalian PARL cleaves Omi/HtrA2, a mitochondrial stress response serine protease,[18,36] remains to be shown, but in the light of the data presented here, a direct role of Hax1 in this processing can be ruled out. Given the peripheral association of Hax1 to heavy membranes as well as its possible calcium-binding capacity (Figure 1) and its interaction with Phospholamban,[37] a sarcoplasmic reticulum protein and a key regulator of Ca^{2+} homeostasis, the reduced level of processed Omi/HtrA2 observed in *Hax1*$-/-$ cells could be explained by the defects in calcium signaling and homeostasis.[38–43] Accordingly, the phenotype of the *Hax1*$-/-$ mouse could be explained by multiorganellar failure due to defective calcium homeostasis. Thus, our study re-opens the question of whether and how the organelle governs PARL activity to regulate mitochondrial stress, a process that can trigger mitochondrial dysfunctions that are central to the etiology of cancer[44–46] and multiple neurodegenerative disorders, including Parkinson's disease.[47,48]

Our study showed that Hax1 is not an IMS protein containing BH modules, thereby ruling out a model in which Bcl-2 proteins mediate the elimination of active Bax from inside the organelle during MOMP. Identification of Bcl-2 family-related proteins is a challenging task because some members of this family show no conserved sequence motifs (at least, not statistically significant ones)[49] and are recognized solely on the basis of structural similarity to *bona fide* Bcl-2-family proteins.[23] However, Hax1 does not meet even the most liberal criteria for the presence of BH modules. First, the secondary structure of Hax1 that we confidently and consistently predicted with three computational methods is incompatible with the presence of such modules and, second, the sequences of Hax1 that were previously aligned with BH1 and BH2 domains are very poorly conserved during the
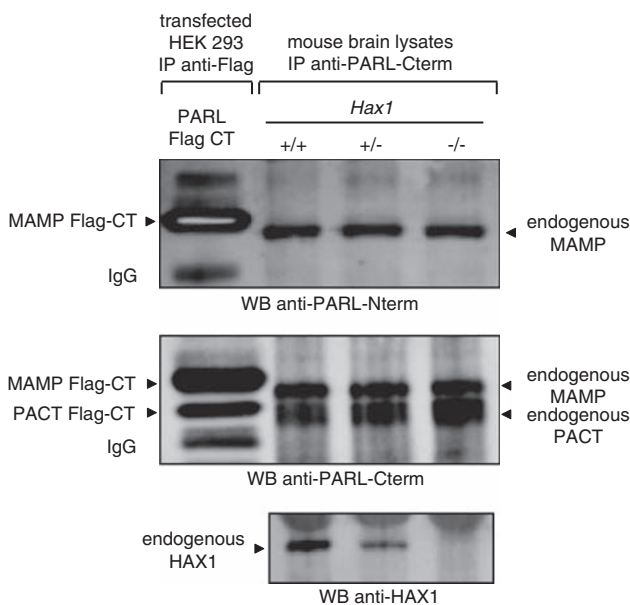
evolution of Hax1, an observation that effectively rules out a key role of these regions in Hax1 function and that is in agreement with reports recently published by other groups.[50] In conclusion, although it could be formally argued that only the crystal structure of Hax1 can put the hypothesis that Hax1 is a member of the Bcl-2 family to final rest, the analysis reported here makes this possibility exceedingly unlikely.

Materials and Methods

Computational analysis of protein sequences. Hax1 orthologs from all the sequenced animal genomes were detected by searching the Genpept database using the PSI-BLAST program,[51] and a multiple alignment of a selected set of diverse sequences was constructed using the T-Coffee program.[52] Secondary structure (SS) prediction was performed using the program PSIPRED,[53] JPRED[54] and PredictProtein (PRPROT).[55] The search for conserved protein domains was performed using the RPS-BLAST program and the Conserved Domain Database.[56,57] Mitochondrial import prediction was done using the MitoProt II program.[58] Transmembrane domains were predicted using PredictProtein,[55] as well as TMHMM[59] and TMPred.[60]

Mitochondria purification. Mitochondria were purified as described.[41] Briefly, mouse livers were washed once with PBS, suspended in isolation buffer (200 mM sucrose, 1 mM EGTA-Tris and 10 mM Tris-MOPS, pH 7.4), and then disrupted by dounce homogenization on ice. The homogenate was spun at $800 \times g$ for 10 min at $4°C$; the supernatant was recovered and further centrifuged for 10 min at $8000 \times g$ at $4°C$. The resulting pellet (mitochondrial fraction) was collected, whereas the supernatant was further spun for 30 min at $100\,000 \times g$ at $4°C$. The resulting pellet (light membrane fraction) and supernatant (cytosolic fraction) were spun again at $100\,000 \times g$ to further purify the fractions. The mitochondrial fraction was purified further by centrifuging twice at $8000 \times g$ for 10 min at $4°C$. The obtained pellet was purified by centrifugation at $95\,000 \times g$ for 30 min on a 30% Percoll gradient in isolation buffer. The mitochondrial layer was washed free of Percoll and resuspended in isolation buffer. Subcellular fractions were also obtained by differential centrifugation from mouse liver and MEFs as described.[61] Protein concentration was determined and the indicated amounts of protein were separated by SDS-PAGE and immunoblotted.

Alkaline extraction. Heavy membranes prepared from HeLa cells were diluted to a final concentration of 1 mg/ml in 20 mM HEPES/KOH (pH 7.4) (hyposmotic buffer). After the addition of an equal volume of freshly prepared 0.2 M sodium carbonate (pH 11.5), samples were incubated for 30 min at $4°C$. The membrane and soluble fractions were separated by ultracentrifugation at $100\,000 \times g$ for 30 min at $4°C$.

Cell culture and transfection. HEK 293T and HeLa cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell types used in this study were maintained under standard cell culture conditions. Cells were transfected at 40% confluence with FuGENE 6 (Roche, Mannheim, Germany). Hax1−/− MEFs and brains were kindly provided by Dr. JN Ihle; Parl−/− MEFs by B De Strooper.

Cloning and mutagenesis. The pcDNA3 vector was used to express the human PARL and mouse Hax1 protein in mammalian cells. Mutants were obtained by site-directed mutagenesis (Clontech, Mountain View, CA, USA). For the mammalian two-hybrid assay, the Checkmate System and vectors (pBind, pACT, pG5luc) were used (Promega, Madison, WI, USA). The identity of every construct and mutant was confirmed by DNA sequencing.

Co-immunoprecipitations. Transfected HeLa cells were lysed in STEN buffer; heavy membranes in CHAPS buffer. Immunoprecipitations were conducted as described.[10,29]

Antibodies. Rabbit anti-PARL;[29,34] rabbit anti-MnSOD (Stressgen, Ann Arbor, MI, USA; 1 : 1000); mouse anti-UQCRC2 (clone 13G12, Molecular Probes, CA, USA; 1 : 3000); mouse anti-HA (Roche); mouse anti-Flag M2 (Sigma, St Louis, MO, USA); mouse anti-actin (clone C4, Cedarlane, Hornby, ON, Canada; 1 : 1000); mouse anti-cytochrome c (clone 7H8.2C12, BD Pharmingen, Mississauga, ON,

Canada; 1 : 1000); mouse anti-Hax1 (clone-52, BD Biosciences, Mississauga, ON, Canada; 1 : 250) and rabbit anti-calnexin (Stressgen, Ann Arbor, MI, USA; 1 : 4000). The polyclonal rabbit anti-Hax1 antibody was raised against peptides encompassing amino acid 126–142 and 201–217 of mouse Hax1 (1 : 500).

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