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The apoptosis mediator mDAP-3 is a novel member of a conserved family of mitochondrial proteins

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SUMMARY

Programmed cell death is essential for organ development and regeneration. To identify molecules relevant for this process, full length cDNA cloning of a short, developmentally regulated murine cDNA fragment, MERM-3, was performed and showed a 1.7 kb mRNA encoding a 45 kDa protein with an ATP/GTP binding motive (P-loop). Sequence analysis revealed an 82% amino acid identity to the human death associated protein 3 (hDAP-3), a positive mediator of apoptosis. The full length sequence being the murine orthologue of hDAP-3 is therefore referred to as mDAP-3. In situ hybridization and northern blot analysis showed an abundant mRNA expression with a pronounced expression in highly proliferative epithelial compartments.

For mDAP-3, cytochrome c release and induction of cell death could be demonstrated by overexpression of a mDAP-3/EGFP fusion protein. DAP-3 mediated apoptosis was shown to depend on a functional P-loop. Intracellular localization studies using the mDAP-3/EGFP fusion protein, cell fractionation and protease protection experiments localized mDAP-3 to the mitochondrial matrix. DAP-3, in contrast to cytochrome c, retained its mitochondrial localization during apoptosis induction.

A mutant of a putative yeast orthologue of mDAP-3, YGL129c, here referred to as yDAP-3, has been shown to exhibit disrupted mitochondrial function. yDAP-3 deficient mutants could be shown to progressively loose mitochondrial DNA. Loss of mitochondrial DNA in yDAP-3 was partially prevented by transfection of the yDAP-3 deficient mutant with mDAP-3, indicating functional complementation by murine DAP-3 in the yeast system.

These data identify mDAP-3 as one of the first proapoptotic factors in the mitochondrial matrix and provide evidence for a critical, evolutionary conserved role of members of the DAP-3 protein family for mitochondrial biogenesis.

Key words: Apoptosis, DAP-3, Development, Mitochondrial matrix, Mitochondrial DNA, P-loop

INTRODUCTION

The process of cellular suicide known as apoptosis or programmed cell death is fundamental to the development and maintenance of all multicellular organisms (Thompson, 1995). Cells undergoing apoptosis show cell shrinkage, membrane blebbing, and condensation and fragmentation of nuclear chromatin (Kerr et al., 1972). Disregulation of this process results in various pathologies including several autoimmune and neurodegenerative diseases and cancer.

Over the last year overwhelming evidence was presented, that mitochondria act as the central cellular organelles for the control of life and death of a cell. Once thought to only generate energy, they are now considered to act as important 'decision centers' involved in programmed cell death (Martinou, 1999). They harbor proteins that trigger apoptosis upon release into the cytosol (Alnemri, 1999). One of these proteins, cytochrome c, shuttles electrons between protein complexes of the respiratory electron transport chain. After release from the mitochondria, cytochrome c participates in the activation of caspases. A flavoprotein known as apoptosis inducing factor (AIF) is normally confined to mitochondria but translocates to the nucleus when apoptosis is induced. Microinjection of AIF into the cytoplasm of intact cells induces condensation of chromatin, dissipation of the mitochondrial transmembrane potential, and exposure of phosphatidylserine in the plasma membrane. Like cytochrome c, AIF is likely to be a phylogenetically old, bifunctional protein with an electron acceptor/donor function and a second, independent apoptogenic role (Susin et al., 1999).

ANT-1 is another member of mitochondrial proteins involved in apoptosis induction. In a recent study it could be shown that ANT-1 overexpression led to all features of apoptosis (Bauer et al., 1999). ANT-1 is a component of the mitochondrial permeability transition complex, a protein aggregate connecting the inner with the outer mitochondrial membrane that has recently been implicated in apoptosis.

The apoptosis mediator, hDAP-3, was isolated through a

functional selection approach in HeLa cells (Kissil et al., 1995). Expression of anti-sense hDAP-3 mRNA protected cells from apoptosis induced by activation of Fas- and tumor necrosis factor α (TNF-α)-receptors. Thus hDAP-3 is implicated as a positive mediator of these death-inducing stimuli (Kissil et al., 1999).

In the present study we report the identification, molecular cloning and initial characterization of a murine protein termed mDAP-3. mDAP-3 was identified by an approach to isolate markers of murine nephrogenesis, based on a modified differential display polymerase chain reaction (Kretzler et al., 1996). mDAP-3 is a putative orthologue to the human DAP-3. Orthotopic overexpression of a mDAP-3/EGFP fusion protein induces apoptosis. Fluorescence microscopy revealed a mitochondrial distribution of the fusion protein. The native mDAP-3 could be localized to the mitochondrial matrix according to its release after digitonin and carbonate treatment from isolated mitochondria.

mDAP-3 showed a significant sequence similarity to a putative yeast orthologue (YGL129c), here refered to as yDAP-3. A yDAP-3 defective yeast strain progressively lost mitochondrial DNA. The loss of mitochondrial DNA was significantly mitigated upon expression of the mammalian mDAP-3.

MATERIALS AND METHODS

cDNA cloning of full-length mDAP-3 cDNA

To clone full length mDAP-3, the EMBL mouse EST database was searched using BLAST (Altschul et al., 1990). A 578 bp mouse EST clone (MM4446) with a 99% sequence identity in a 100 bp overlap with MERM-3 was identified. This sequence was used to screen 3×10^6 clones of a λ-ZAP cDNA library of E17.5 (embryonic day 17.5 post conception) mouse kidneys (Valentini et al., 1997) as described earlier (Church and Gilbert, 1984). The two longest of seven independent clones were bi-directionally sequenced by primer walking. The 5′ sequence of the mDAP-3 cDNA was verified by 5′-Marathon PCR (Clonetech, Heidelberg, Germany) in three independent bi-directionally sequenced clones, giving a full length cDNA of 1,656 bp.

Sequence analysis

The Heidelberg Unix Sequence Analysis Resource (HUSAR) was used to assemble the full-length consensus sequence of the mDAP-3 cDNA. For database searches the BLASTN program was applied at the nucleotide and the BLASTP program at the protein level. The PROTEIN and PROSITE programs were used to analyze the deduced mDAP-3 protein and potential motives, respectively. The PSORT-program (http://psort.ims.u-tokyo.ac.jp/) identified the putative intracellular localization.

In situ hybridization

Single-stranded radiolabeled riboprobes for mDAP-3 were prepared by in vitro transcription of a 286 bp PCR fragment, corresponding to position 334 to 619 of the mDAP-3 consensus sequence and hybridized as described earlier (Angerer and Angerer, 1992; Simmons et al., 1989). The ^{35}S-labeled antisense and sense RNA transcripts served as hybridization probe and control, respectively. After hybridization and development, the tissue was counterstained with Harris hematoxylin and eosin and examined on a microscope with dark and bright field optics.

Northern blot

For northern blot analysis total RNA was isolated from adult mouse tissue by guanidine thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). After separation and immobilization of 20 μg total RNA on nylon membranes, hybridization was performed with the 578 bp long mDAP-3 riboprobe also used for library screening. For detection of signal the Storm Phosphorimaging System (Molecular Dynamics, Krefeld, Germany) was used. To assess the relative quantity of mRNA per lane, the organ screen blot was stripped and re-probed with a mouse β-actin probe.

Cell lines and transient transfections

Mouse tubular cells (MTC) and mouse mesangial cells (MMC) were cultured as described earlier (Haverty et al., 1988; Wolf et al., 1992). Jurkat cells were supplied by ATCC and cultured in RPMI with 10% fetal calf serum and 1% penicillin/streptomycin. For transient transfections, cells were harvested with trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA 4Na; Life Technologies, Inc., Karlsruhe, Germany), resuspended in PBS to a concentration of 2.5×10^6/ml and electroporated at 250 V and 960 μF with 1-10 μg of plasmid DNA. Following transfection cells were cultured under normal conditions for 24-48 hours prior to further analysis.

Western blot

MMC and MTC were harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholat, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 μg/ml pepstatin, 1 μg/ml leupeptin). Extracted proteins or isolated mitochondria (see below) were boiled in loading buffer for 10 minutes, resolved by SDS-PAGE under reducing conditions and transferred to an Immobilon-P membrane (Millipore, Eschborn, Germany). Blots were probed with the respective primary antibodies and immune complexes were visualized using enhanced chemoluminescence (ECL-kit, Amersham International, Braunschweig, Germany).

The following antibodies were used: DAP-3 (Transduction Laboratories, Lexington, KY); cytochrome c (Santa Cruz Biotechnology Inc., Heidelberg, Germany). HSP60 (heat shock protein 60) and AAC (ADP/ATP carrier) antibodies were kind gifts from W. Neupert (Physiologic Chemistry, University of Munich, Germany).

Plasmid construction

mDAP-3-enhanced green fluorescence fusion protein (mDAP-3/EGFP) was obtained by cloning a PCR fragment containing nucleotides 1-1342 of mDAP-3 into the *Bam*HI/*Sal*I site of the vector pEGFP-N1 (Clonetech, Heidelberg, Germany). The mDAP-3 stop codon at position 1342-1345 was changed from the nucleotides TAA that code for the translation stop to the nucleotides TCA which code for the amino acid serine. The mDAP-3 coding region is then followed by 6 (7 including the changed stop codon) linker codons originating from the pEGFP-N1 vector multiple cloning site and the EGFP ATG translation start codon, that marks the beginning of the EGFP open reading frame.

A mDAP-3 P-loop mutant was obtained by site directed mutagenesis of amino acid 127 from lysine to arginine with the Quick Change™ Site-Directed Mutagenesis Kit as recommended by the manufacturer (Stratagene, Amsterdam, The Netherlands).

For the expression of mDAP-3 in yeast, a PCR fragment containing nucleotides 151 to 1345 of the mDAP-3 cDNA was subcloned into the *Eco*RI/*Sal*I site of the yeast expression vector pYX 123 (Novagen, Madison, USA).

Cell death assay

MMCs were transfected by electroporation with 9 μg of the indicated plasmids (pEGFP-N1 or mDAP-3/EGFP) together with 1 μg of a plasmid coding for farnesylated green fluorescent protein (fGFP). fGFP as control was required do to leaching of the pEGFP-N1 after ethanol fixation. For FACScan analysis cells were detached 48 hours after transfection with trypsin/EDTA, washed with PBS,

and fixed in 50% ethanol for at least 30 minutes at room temperature. After washing with PBS, cells were treated with RNase A (50 μg/ml) for 30 minutes at room temperature and stained with propidium iodide (PI) for 20 minutes on ice. Cells were subjected to flow cytometry analysis with a FACS-Calibur (Becton Dickinson, Mountain View, CA), where green fluorescent protein (GFP)-positive cells were gated by their high fluorescent intensity (FL1). For each sample, 10,000 GFP-positive cells were collected and cell-cycle distribution was analyzed according to DNA content using PI staining. The results represent three independent transfections. Jurkat cells were stimulated with 50 ng/ml anti-human Fas antibody (Biomol, Hamburg, Germany) for up to 5 hours and prepared for FACScan analysis as described above. Control cells received carrier (PBS) only.

Fluorescent light and confocal laser microscopy

To assess mitochondrial and lysosomal morphology, fluorescent dyes were utilized according to the manufacturer's suggestions. In brief: MTCs or MMCs were grown on glass coverslips for 24 hours after transient transfection with mDAP-3/EGFP and then stained with the appropriate cell culture medium containing either Mito-Tracker-Red CMXRos™ or Lyso-Tracker-Red™ (Molecular Probes, Leiden, The Netherlands) at a concentration of 100 nM for 40 minutes at standard cell culture conditions. Subsequently cells were rinsed 3 times with PBS, fixed with 4% paraformaldehyde and embedded in Mowiol solution (Calbiochem-Novabiochem, Bad Soden, Germany) and visualized by fluorescence microscopy. For confocal laser scanning microscopy cells were analyzed after fixation with 4% paraformaldehyde in PBS.

Cell fractionation

Using an established protocol for isolating mitochondria by differential centrifugation (Werner and Neupert, 1972), mitochondria were isolated from adult rat liver. Lactate dehydrogenase, malate dehydrogenase and glucose 6-phosphat dehydrogenase measurements were used to confirm effective cell fractionation.

Subcellular fractionation of MTCs and Jurkat cells

Mitochondrial and cytosolic fractions were obtained by differential centrifugation of MTCs and Jurkat cells. In brief, cells were harvested and washed in PBS, and resuspended in 5 mM HEPES, 2 mM $MgCl_2$, 75 mM NaCl, 5 mM EDTA and protease inhibitors. Cells were broken by multiple passages through a 25GA1 0.5×25 needle. The suspension was centrifuged at 2,000 ***g*** at 4°C for 5 minutes to remove unbroken cells. The supernatant was centrifuged at 15,000 ***g*** at 4°C for 20 minutes. The resulting supernatant (cytosol) and pellet (mitochondria rich pellet) were analyzed by SDS/PAGE immunoblotting (30 μg per lane).

Mitochondrial membrane permeabilisation with digitonin

Isolated mitochondria were incubated with increasing digitonin concentration, from 0% to 0.4% (see Fig. 5) for 2 minutes on ice. After incubation, the mitochondria were pelleted as above, resuspended in loading buffer, and supernatant and resuspended mitochondria were submitted to western blot analysis.

Protein fractionation by alkaline treatment

Isolated mitochondria (0.2 mg) were pelleted at 7,000 ***g*** for 10 minutes, resuspended in 1 ml 0.1 M Na_2 HCO_3, and incubated at 4°C for 30 minutes. The samples were split and one half was directly trichloracetate (TCA) precipitated with 0.5 ml 70% TCA, representing the total protein content. The second half was centrifuged for 60 minutes at 4°C and 200,000 ***g***. The pellet containing integral membrane proteins was directly resolved in loading buffer. The supernatant containing the soluble proteins and membrane associated proteins was trichloracetate precipitated. All three fractions were then submitted to western blot analysis.

Characterisation of yDAP-3 and complementation with mDAP-3

Standard techniques were used for the growth and manipulation of yeast strains (Guthrie and Fink, 1991). A yDAP-3 (YGL129c) null strain was a kind gift from E. Cerdan (La Coruña, Spain; Tizon et al., 1999). This mutant did not contain mitochondrial DNA and was backcrossed with a YPH500 wild-type strain (Sikorski and Hieter, 1989). Sporulation revealed yDAP-3 null mutants containing mitochondrial DNA. These strains were used for the experiments shown. To test the stability of the mitochondrial genome, yeast cultures were grown for various times in liquid glucose medium prior to crossing with a tester strain lacking mitochondrial DNA. Respiration competence of the resulting diploids was tested on glycerol plates.

RESULTS

Cloning and identification of mDAP-3

The MERM-3 (for mouse embryonic renal marker 3) cDNA fragment had been initially identified through a modified differential display polymerase chain reaction (Kretzler et al., 1996). Semiquantitative RT-PCR was used to reconfirm the MERM-3 expression pattern and to analyze MERM-3 mRNA levels during in vivo and in vitro nephrogenesis. MERM-3 was found to be upregulated during embryogenesis (Kretzler et al., 1996). Northern blot analysis of either adult or E17.5 mouse kidney mRNA hybridized with a 578 bp riboprobe gave a specific signal for mDAP-3 indicating a length of about 1.7 kb for the mDAP-3 mRNA (data not shown). The same probe was used to screen an E17.5 days p.c. mouse kidney cDNA library and a full-length cDNA of 1656 bp was obtained. These sequence data have been submitted to the EMBL database under accession number: AJ250375. The mDAP-3 cDNA sequence contains a single open reading frame (ORF) coding for a potential protein of 391 amino acids with a calculated molecular mass of about 45 kDa. No potential transmembrane regions were identified by hydrophobicity blot.

The mDAP-3 sequence contains a typical P-Loop motive, that fits to the consensus sequence (G)XXXXGK(S/T) at positions 121-128, which implies that mDAP-3 is a potential ATP/GTP-binding protein. In addition mDAP-3 contains multiple putative PKC- and CK2-phosphorylation sites. The N terminus of mDAP-3 is positively charged and could serve as a mitochondrial targeting sequence (Nakai and Horton, 1999).

Tissue distribution of mDAP-3

In situ hybridization with mDAP-3 specific riboprobes showed an abundant expression in mouse embryo (Fig. 1A). The corresponding sense probe gave no specific signal (Fig. 1A, inset). Strong signal was detected in the developing epithelial compartments, particularly of the oropharynx, the gastrointestinal tract, dermis and the urogenital system. The central nervous system, the cerebral cortex and the trigeminal nerve showed prominent expression. Maximal expression was found in the derivatives of the pharyngeal pouch (Glandulae thyreoidea and subingualis) with highest mRNA levels in the olfactorial epithelium.

Northern blot analysis of 20 μg total RNA from 11 organs of adult mice showed mDAP-3 mRNA expression in the kidney, heart, liver, thymus, muscle, spleen, intestine and stomach, with an expression maximum in the testis (Fig. 1B).

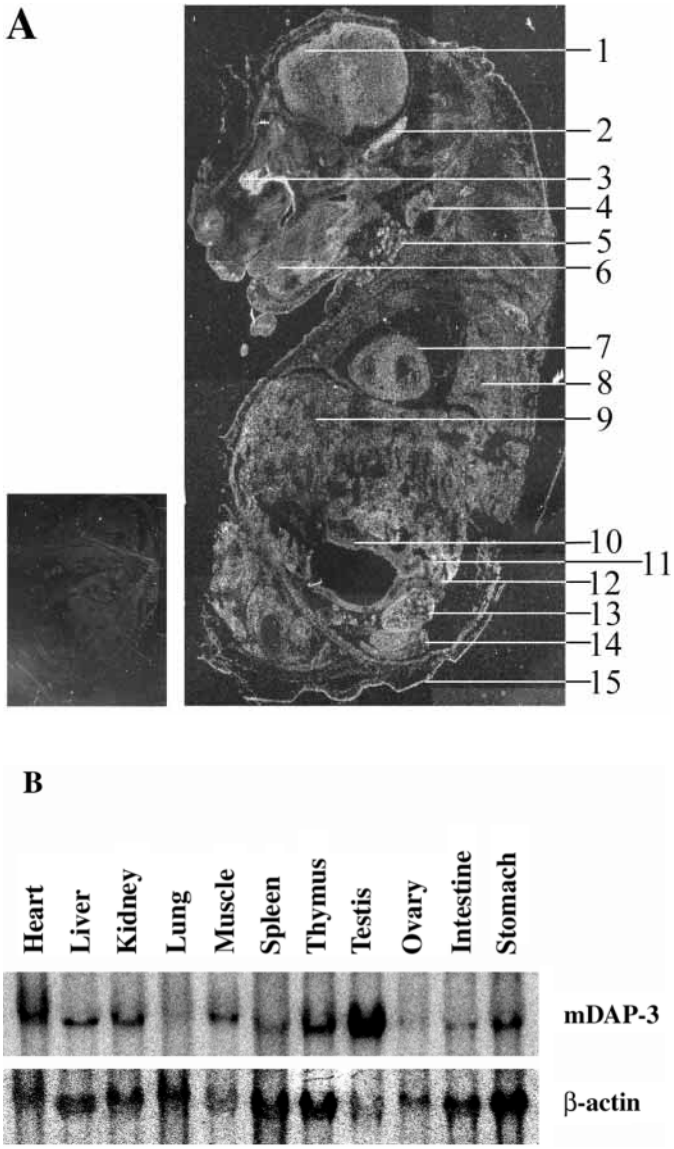

Fig. 1. Tissue distribution of mDAP-3. (A) In situ hybridization of a median section from a mouse embryo 17.5 days post conception, using a ^{35}S labeled 286 bp mDAP-3 antisense riboprobe, darkfield image, 4× magnification. Structures displaying hybridization signal correspond to:1: Cerebral cortex, 2: Trigeminal nerve, 3: Olfactorial epithelium, 4: Gld. thyroidea, 5: Gld. sublingualis, 6: Musculi linguae, 7: Heart, 8: Lung, 9: Liver, 10: Stomach, 11: Small intestine, 12: Adrenal gland, 13: Kidney, 14: Gonad, 15: Epidermis. Insert: Sense control with low background signal. (B) Northern blot of 20 μg adult mouse total RNA of the indicated organs probed with a 578 bp riboprobe. Upper panel: 578 bp mDAP-3 probe; lower panel: β-actin control hybridization. mDAP-3 expression is found in a wide variety of tissues with an expression maximum in testis.

Minimal to absent expression was seen in ovary and lung, respectively.

Amino acid sequence comparison of mDAP-3, hDAP-3, *Caenorhabditis elegans* DAP-3 and *Saccharomyces cerevisiae* DAP-3

By searching the EMBL protein database with a BLAST algorithm using the full length mDAP-3 cDNA as the query, the DNA sequence for the hDAP-3 was retrieved. A nucleic acid comparison of mDAP-3 and hDAP-3 showed an 82% identity over 1299 base pairs. The amino acid identity between the 391 amino acid mDAP-3 protein and the 398 amino acid hDAP-3 protein is 82% (Fig. 2A). Alignment of the *C. elegans* cec14a_4.2 with mDAP-3 shows 33% identity between the proteins (Fig. 2A). In addition, in a search of the yeast genome, an open reading frame (YGL129c) with a 17% identity with mDAP-3 (including the P-loop region) was found and is referred to as yDAP-3 (Fig. 2A). Screening the mouse EST database at the NCBI with the human DAP-3 sequence revealed 59 matching murine ESTs (P<0.01). Using the consensus sequence generated from those ESTs a mDAP-3 identical ORF could be deduced, further supporting the notion of mDAP-3 being the murine orthologue of hDAP-3.

By searching the EMBL protein database using only the mDAP-3 P-loop sequence as a query, two additional proteins were retrieved. C.elegans protein Ced-4 (cell death gene 4) and the mammalian orthologue to Ced-4, Apaf-1. An alignment of the P-loop sequences of these proteins (Fig. 2B) shows that the homology exceeds the consensus sequence defining a P-loop motive XXGXXXXGK(S/T), giving a consensus sequence of **L**XGXX**G**XGK(S/T).

Functional analysis of mDAP-3 by transient overexpression

To test whether mDAP-3 has an effect on cell survival, MMCs were transiently transfected with a mDAP-3/EGFP fusion protein or the empty expression vector.

Expression of the mDAP-3/EGFP fusion protein was verified by western blot analysis using an anti-hDAP-3 antibody. Two distinct bands were detected, the lower 45 kDa signal represents the endogenously expressed mDAP-3 and the 75 kDa band corresponds to the mDAP-3/EGFP fusion protein (Fig. 3A), confirming transient expression of mDAP-3 and cross-reactivity of the hDAP-3 antibody.

The mDAP-3/EGFP overexpression experiments were designed as co-transfections with a vector expressing a membrane localized form of GFP also used in controls. The cells were fixed with ethanol 48 hours after transfection, stained with PI and analyzed by FACScan for their DNA content and the overall cell survival. Apoptosis was assessed by determing the number of cells within the GFP positive gated population with a sub G_1 DNA content, indicative of DNA fragmentation.

mDAP-3/EGFP fusion protein overexpression increased the number of MMCs displaying a sub-G_1 DNA content from a background of 11.9±2.8% to 27.6±2.6% apoptotic cells (n=3) (Fig. 3B). The cell cycle distribution of the viable cells did not change significantly. Thus mDAP-3 expression in MMCs induced an increase in the number of apoptotic cells.

To further dissect the pro-apoptotic effect of DAP-3, transient transfection of wild type mDAP-3 and a construct with a point mutation in the P-loop (lysine 127 changed to arginine 127) were analyzed. P-loop mutation resulted in an abolishment of the proapoptotic effect of mDAP-3 (wild-type mDAP-3: 25%, P-loop mutant mDAP-3: 4.1%, CON 8.6% sub-G_1 DNA content). In the mDAP-3 transfected cells cytochrome c could be detected in the cytoplasm by cell

A

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mDAP-3    ............................................................   0
hDAP-3    ............................................................   0
ceDAP-3   ............................................................   0
yDAP-3    MYKNIYICILYIYINFYTSRENINKRTSTSHVVIRIRIMLRMSTSRFIGQRLFTTARSLQ  60

mDAP-3    .................MLTGITRLFSRVQKLDPRCFLHMSVQATQ......NSQVPAER  37
hDAP-3    ................MMLKGITRLISRIHKLDPGRFLHMGTQARQSIAAHLDNQVPVES  44
ceDAP-3   ................................MKPCGNLMCQIARR.......LPARQLH  21
yDAP-3    AAKPAPKGKTQGFSKKSSSVSSYSSAKRVTPGSLYKNWTNTTHTAQLQQTAVPLALPIFN 120

mDAP-3    PRTVSRTSDSDPAKHGEQHEGQHYSIPLQDLKTVFPHGLPPRYMMQVKTFGEACLMVRKP  97
hDAP-3    PRAISRTNENDPAKHGDQHEGQHYNISPQDLETVFPHGLPPRFVMQVKTFSEACLMVRKP 104
ceDAP-3   ...ISRRSNSQLADVGTLSEIEMP....VAKQLNMRNHLPAPLSKQMDTLGEVVTLIREP  74
yDAP-3    FDDISKTLNKVVSYSNKQYKSLHHLGSFKKSQFNELFQKPVCLVREDATNSFLKKLV... 177

mDAP-3    ALELLGYLKNTNFAHPAVRYLLYGEKGTGKTLSLCHAVHFCARHDWLILHIPDAHLWVKN 157
hDAP-3    ALELLHYLKNTSFAYPAIRYLLYGEKGTGKTLSLCHVIHFCAKQDWLILHIPDAHLWVKN 164
ceDAP-3   MAEVASCMRVVDKSLPALRLMLWGAFGTGKSVTLNQAVHHAYNNKWAIVHLRSAMELTRR 134
yDAP-3    .............SHPVKKFIITGEPGVGKTVLLSQAHAYAVDSKQIIINISYPELFLNG 224

mDAP-3    CRELLQSTHNKQRFDQPLEASTWLKNFKTTN...ERFLSQIKVQEKYVWN.......... 204
hDAP-3    CRDLLQSSYNKQRFDQPLEASTWLKNFKTTN...ERFLNQIKVQEKYVWN.......... 211
ceDAP-3   VKEIEMSSFVQGRINDPSNSVSILQNFKQQNQHIWKILGELLTERDYEWS.......... 184
yDAP-3    RNDFSYDDDLKLFIQPMYLKKLIRKILKANDP..A.LLKSIELSKDYKFSNANPKNASVK 281

mDAP-3    KRESTEKGSPLGEVVEQGLTRVRNATDAVGVVLKELKAQSALGLFHLLVAVDGVNALWGR 264
hDAP-3    KRESTEKGSPLGEVVEQGITRVRNATDAVGIVLKELKRQSSLGMFHLLVAVDGINALWGR 271
ceDAP-3   KSERTLKGKPITEIIEMGISAPFLASDCVGAIFRELRRHAKDDKLKVLVAIDDANSLWGK 244
yDAP-3    PFVTLNKTKNTVLDLLSVMTHPHNRGKLMKAIIDELSVQS.....KVPIMFTVDNFSKVL 336

mDAP-3    TTLKKEDRTLIAPEELSLVHNLRKMVKNDWHGGAIVLSLSQTGSLFK....SRTAYLPHE 320
hDAP-3    TTLKREDKSPIAPEELALVHNLRKMMKNDWHGGAIVSALSQTGSLFK....PRKAYLPQE 327
ceDAP-3   TLVKRADRTYAPPSDLTLVVHYRRMIENDWTNGCVLMVADKKEVADARDHLGVSRHTPLE 304
yDAP-3    TTAYSAYRNTENKQIYSLDLQMGKLMMDIISGETKFANGESSTILAISGVDRTNKTLPVA 396

mDAP-3    LLGKEGFNALEP...............FLPILIPNYNPKEFESSFQYYLENNWLQHEKAS 365
hDAP-3    LLGKEGFDALDP...............FIPILVSNYNPKEFESCIQYYLENNWLQHEKAP 372
ceDAP-3   LFGEEGFEYVEP...............FVPIETSNYTEAETDAIYNYYVNKNWLASKAAR 349
yDAP-3    LGKIPVDPYVTRYHYEPKFVELLQKGNVTEFEVPKLNKQEVNELIDYYKQSNVLLDKDIT 456

mDAP-3    ...TEEGRKELRFLSNCNPEQLERLCASL                                391
hDAP-3    ...TEEGKKELLFLSNANPSLLERHCAYL                                398
ceDAP-3   ...SEEGRKQLMHLSAFNPYYYERLSEVRSASQWIKASNSKTPDDINVEKLFVE       400
yDAP-3    GKKWENLIDEKYFLSGNGNPRELLKSLVLSHR                             488
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B

	X	X	**G**	X	X	X	X	**G**	**K**	**[TS]**		P-loop consensus
119	**L**	**Y**	**G**	E	K	**G**	**T**	**G**	**K**	**T**	128	mDAP-3
126	**L**	**Y**	**G**	E	K	**G**	**T**	**G**	**K**	**T**	135	hDAP-3
096	**L**	**W**	**G**	A	F	**G**	**T**	**G**	**K**	**S**	105	ceDAP-3
141	**I**	**H**	**G**	N	A	**G**	**C**	**G**	**K**	**S**	150	Apaf-1
157	**L**	**H**	**G**	R	A	**G**	**S**	**G**	**K**	**S**	166	CED-4
186	**I**	T	**G**	**E**	P	**G**	V	**G**	**K**	**T**	195	yDAP-3

Fig. 2. Amino acid comparison of mDAP-3, hDAP-3, *C. elegans* DAP-3 and yDAP-3. (A) Comparison of the mDAP-3 and the DAP-3 human, nematode and yeast protein sequences. The mDAP-3 protein shows an identity of 82% to the human, 33% to *C. elegans* and 17% to the yeast DAP-3 orthologues. Identities are shaded and similarities are boxed. (B) Alignment of the P-loop consensus sequence and the mDAP-3, hDAP-3, ceDap-3, Apaf-1, Ced-4 and yDAP-3 P-loop sequences. Similar or identical amino acids are printed bold. The P-loop homology between these proteins exceeds the overall consensus sequence, consistent with a similar P-loop function for these proteins, but probably in a different context.

fractionation assays (Fig. 3C). In the P-loop mutants and controls no cytochrome c release could be shown (Fig. 3C). Furthermore, mDAP-3 remained intra-mitochondrial after apoptosis induction (Fig. 3C). These data indicate the functional requirement of the P-loop for mDAP-3 mediated apoptosis, and locate DAP-3 upstream of cytochrome c release during cell death.

Subcellular localization of mDAP-3

To determine the intracellular localization of mDAP-3, the mDAP-3/EGFP expression plasmid was employed. Examining transiently transfected MTCs and MMCs (data not shown) by fluorescence light microscopy allowed detection of a punctate cytoplasmic signal (Fig. 4B). This pattern was distinctly different from the even and diffuse fluorescence derived from the expression of the pEGFP-N1 vector alone (Fig. 4A).

To further analyze the punctate intracellular localization of mDAP-3, double labeling studies with the mDAP-3/EGFP fusion protein and cell organelle specific dyes, Mito-Tracker-Red CMXRos™ for mitochondria or Lyso-Tracker-Red™ for lysosomes, were performed. By light microscopy the mDAP-3/EGFP fusion protein colocalized with the mitochondrial dye (Fig. 4C+D) but not with the lysosomal marker (Fig. 4E-F). These results were confirmed by confocal scanning laser microscopy (Fig. 4G-I). Merging the fluorescence of the mitochondrial dye and the mDAP-3/EGFP fusion protein revealed a significant overlap of the signals, consistent with a mitochondrial localization of the mDAP-3/EGFP fusion protein.

Localization of mDAP-3 by cell fractionation

Cell fractionation assays were performed to examine the subcellular distribution of mDAP-3 biochemically. Mitochondrial, endoplasmatic reticulum and cytosol fractions were isolated from rat liver and subsequently tested for their mDAP-3 content by western blot analysis. The fractionation was controlled by measuring compartment specific enzyme activity (data not shown). mDAP-3 protein was only detectable in the mitochondrial fraction consistent with the mDAP-3/EGFP data (Fig. 5A).

Proteinase K treatment of the mitochondrial fraction did not change the amount of mDAP-3, indicating an intramitochondrial localization of mDAP-3 (Fig. 5B).

To further dissect intramitochondrial localization of mDAP-3, sequential disruption of mitochondrial membranes with digitonin was performed. Digitonin treatment lead to a concentration dependant release of mDAP-3. As controls, cytochrome c, localized in the intermembrane space of mitochondria, ADP/ATP carrier (AAC), an integral protein of

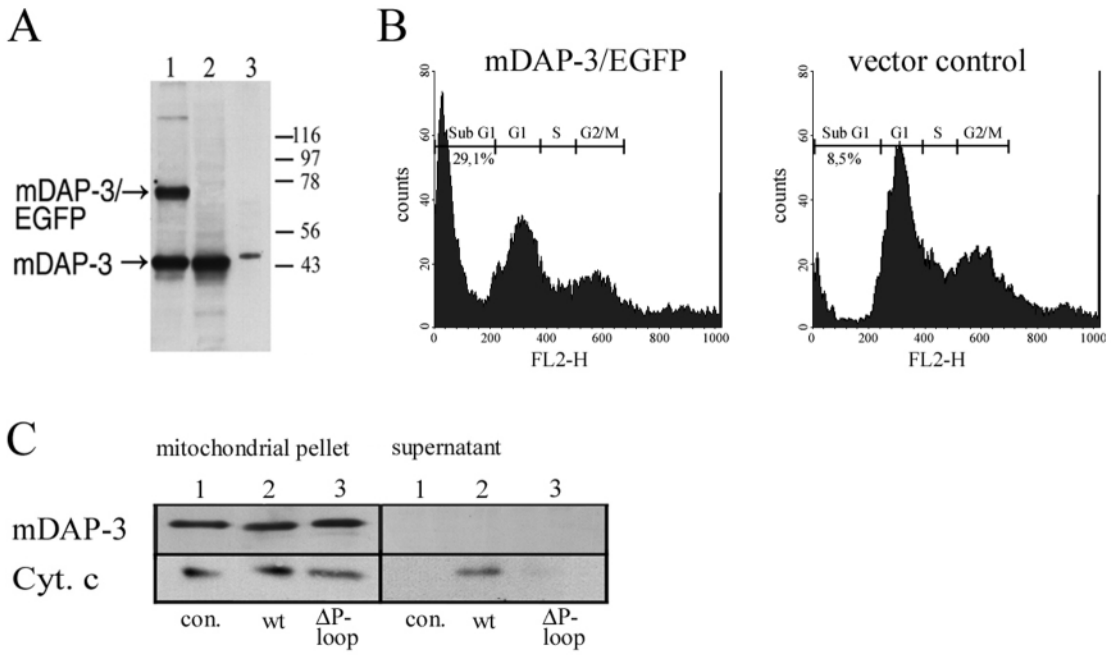

Fig. 3. (A) Western blot of transient overexpression of the mDAP-3/EGFP fusion protein in MMCs; 20 μg of cell lysate per lane were probed with a hDAP-3 antibody. lane 1: mDAP-3/EGFP fusion protein; lane 2: pEGFPN1 expressing only EGFP; lane 3: human sw13 cell lysate as positive control as supplied by the antibody manufacturer. The lower 45 kDa signal represents the endogenously expressed mDAP-3 (expected molecular mass 45 kDa) and the 75 kDa band in lane 1 corresponds to the mDAP-3/EGFP fusion protein, confirming the detection of the murine mDAP-3 by the human DAP-3 antibody. (B) Overexpression of the mDAP-3/EGFP fusion protein induces apoptosis in MMCs. DNA content distribution of MMCs transfected with farnesylated GFP and either mDAP-3/EGFP fusion protein or an empty vector control. Histograms represent cell counts versus DNA content (FL2-H). The different cell-cycle phases and fractions of cells containing sub-G_1 DNA content are indicated. (C) Western blot of mitochondrial and cytosolic fractions of MTCs. 40 μg per lane were probed with a hDAP-3 or a cytochrome c antibody, respectively. Lane 1: controls (8,6% apoptosis); lane 2: wild-type mDAP-3/EGFP fusion protein (25% apoptosis); lane 3: P-loop point mutated mDAP-3/EGFP fusion protein (4,1% apoptosis). Only cells transfected with the mDAP-3/EGFP fusion protein show cytochrome c release from mitochondria, as seen in supernatant lane 2. mDAP-3 is retained in the mitochondria in all cases.

the inner mitochondrial membrane, and HSP60, localized to the mitochondrial matrix, were used.

As shown in Fig. 5C, mDAP-3 was released late from the mitochondria at 0.3% digitonin, as was AAC (0.3%) and HSP60 (0.2%). Cytochrome c was already detectable in the supernatant after treatment with 0.075% digitonin. These data are consistent with a mDAP-3 localization in the mitochondrial matrix similar to HSP60 or in the inner mitochondrial membrane as the marker AAC. Thus, mDAP-3 is not localized in the intermembrane space as indicated by the different pattern of release by digitonin in comparison to cytochrome c (Fig. 5C).

The mDAP-3 amino acid sequence does not contain hydrophobic stretches. To further analyze the submitochondrial localization carbonate treatment was used which allows to distinguish between soluble and integral membrane proteins. mDAP-3 was found in the soluble fraction, as was cytochrome c and HSP60. AAC was detected in the membrane fraction as expected (Fig. 5D).

Jurkat cells were stimulated to undergo apoptosis with an agonistic anti-Fas antibody and harvested at 3 hours (45% apoptotic cells) and 5 hours (54% apoptotic cells) after stimulation. Subsequent differential centrifugation separated cytosolic and mitochondrial fractions which were then analyzed for their DAP-3, HSP60, AAC and cytochrome c content by SDS/PAGE immunoblotting. DAP-3 as well as the two other mitochondrial matrix or intramembrane proteins HSP60 and AAC could only be detected in the mitochondrial fraction (Fig. 5E). In contrast, cytochrome c could be seen in the cytosol of cells undergoing apoptosis (Fig. 5E).

In summary, these data identify DAP-3 as a mitochondrial matrix protein excerting it's proapoptotic effect inside the mitochondrion.

Characterisation of the yeast DAP-3 orthologue

Since the DAP-3 family is conserved among eukaryotes and has orthologues in non-apoptotic organisms such as yeast, DAP-3 proteins obviously perform an additional role besides the mediation of apoptosis. The localization of DAP-3 in mitochondria lead us to test whether DAP-3 orthologues are required for the biogenesis of this organelle. *S. cerevisiae* appeared to us to be the best suited system to test this non-apoptotic function since the absence of apoptosis in yeast simplifies the interpretation. Disruption of the yDAP-3 gene did not lead to a reduced growth on non-fermentable carbon sources such as glycerol or lactate. This indicates that mitochondrial respiration is not dependent on yDAP-3 function. However, in the absence of yDAP-3, yeast cells were significantly defective in the maintenance of the mitochondrial genome (46% in ΔDAP-3 vs 3% in wild type) (Fig. 6A). Although the molecular role of yDAP-3 remains unclear, this indicates a role of yDAP-3 in mitochondrial

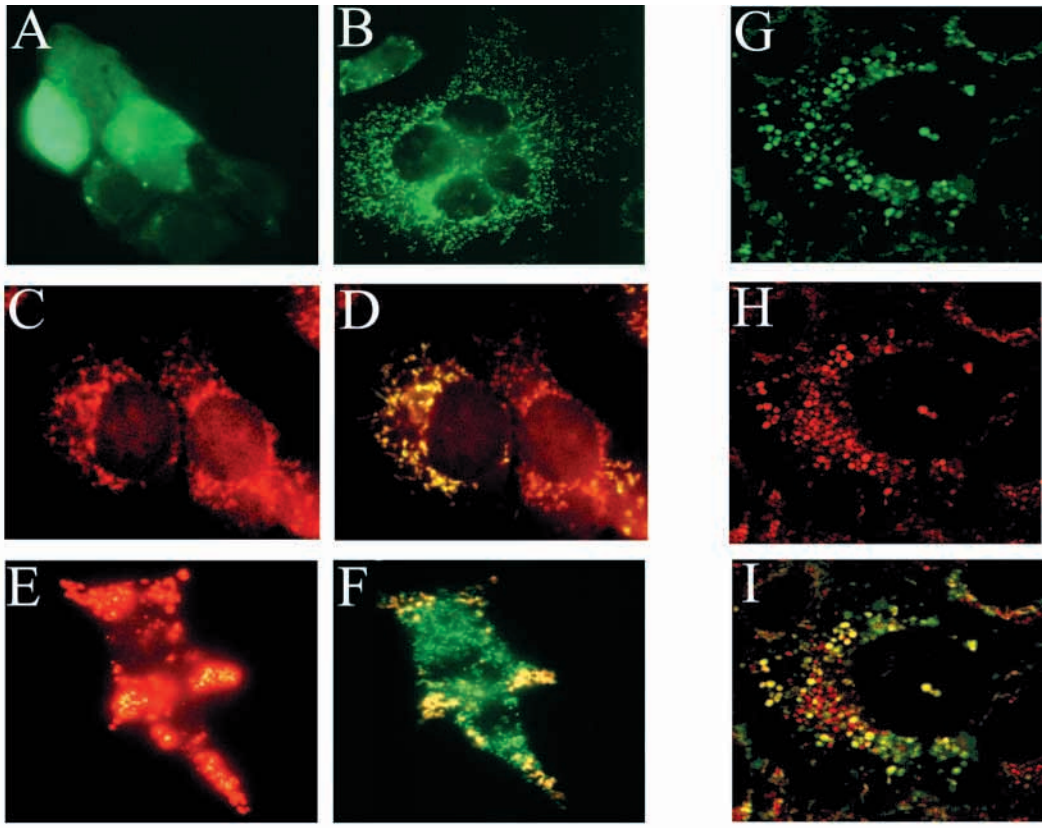

Fig. 4. Localization of mDAP-3. Fluorescence light microscopy images of MTCs transiently transfected with pEGFPN1 expressing only EGFP (A) or the mDAP-3/EGFP fusion protein (B-F). (A) The expected diffuse cytoplasmic signal of EGFP. In B mDAP-3/EGFP, in contrast, displays a punctate signal. In C and D cells were additionally stained with Mito-Tracker-Red CMXRos™ labeling mitochondria. When using the bandpass filter for the GFP signal, the green fluorescence of the fusion protein is completely overlapped by the red fluorescence of the mitochondria specific dye resulting in an orange signal in the one cell positive for the mDAP-3/EGFP fusion protein (D, left cell). The second cell which is not positive for the mDAP-3/EGFP fusion protein does not show an orange signal but retains a weakened red signal resulting from the Mito-Tracker-Red CMXRos™ alone. In E and F, as a negative control, a mDAP-3/EGFP transfected cell was stained with the Lyso-Tracker-Red™ labeling lysosomes. When using the bandpass filter specific for the GFP signal the green fluorescence of the fusion protein remains visible together with the weakened lysosomal fluorescence, indicating that the Lyso-Tracker-Red™ signal does not overlap with the signal of the fusion protein (F). (G to I) Confocal laser scanning images of a paraformaldehyde fixed MTC transfected with mDAP-3/EGFP and stained with Mito-Tracker-Red CMXRos™. (G) The cell stimulated with one laser at 488 nm and the emission measured below 515 nm specific for the mDAP-3/EGFP fusion protein. (H) The same cell stimulated with a second laser at 543nm and the emission measured at 600 nm specific for the Mito-Tracker-Red CMXRos™. The lower picture (I) shows the merged image of G and H. The green fluorescence of mDAP-3/EGFP overlaps with the Mito-Tracker-Red CMXRos™ signal (yellow signal), confirming the mitochondrial localization of the mDAP-3/EGFP fusion protein.

biogenesis. To test whether mammalian DAP-3 orthologues perform a similar role as yDAP-3, we expressed mDAP-3 in yeast. mDAP-3 was efficiently processed to its mature size indicating import of the mammalian orthologue into yeast mitochondria (Fig. 6C). The tendency to loose mitochondrial DNA in yDAP-3 null mutant was significantly mitigated upon expression of the mouse DAP-3 orthologue (Fig. 6B) and thus, the function of DAP-3 homologues in the biogenesis of mitochondria seems to be conserved among eukaryotes.

DISCUSSION

Here we report the isolation and initial molecular characterization of the mDAP-3 full-length cDNA. Northern blot organ screen and in situ hybridization showed a widespread expression with induction in metabolic active tissues. The abundant expression and high evolutionary conservation of mDAP-3 is consistent with the involvement of this molecule in basal cell activities. Given its mitochondrial localization, it can be speculated that mDAP-3 is involved in oxidative metabolism and that the expression induction seen in the in situ hybridization in specific compartments with high mitotic activity mirrors the increased metabolic demand of these cells.

The 1.7 kb mRNA of mDAP-3 contains a single ORF coding for a 45 kDa protein that shares an identity of 82% to the human DAP-3 and 33% identity to the recently identified *C. elegans* DAP-3. hDAP-3 is required for mediation of TNF-α- and Fas induced cell death. A nucleotide binding motive (P-loop) defective mutation of hDAP-3 was unable to induce apoptosis (Kissil et al., 1999). Using a panel of N- and C-terminal truncations preliminary results indicate a requirement of the initial 100 N-terminal amino acids for mitochondrial targeting and/or apoptosis induction by mDAP-3. A P-loop point mutated mDAP-3 is localized to the mitochondria (data not shown), but did not induce apoptosis, consistent with ATP processing by DAP-3 in the mitochondrial matrix being relevant for its proapoptotic activity.

hDAP-3 mediated apoptosis was shown to be suppressed by caspase inhibitors. Dominant negative forms of either FADD/MORT-1 or caspase-8 could not inhibit hDAP-3 induced cell death (Kissil et al., 1999). DAP-3 overexpression resulted in a P-loop dependant release of cytochrome c from mitochondria. These results place molecules of the DAP-3

family downstream of caspase-8 and upstream of cytochrome c and effector caspases, exactly where the decision center between ‘life and death’, the mitochondrion, channels into the pathway.

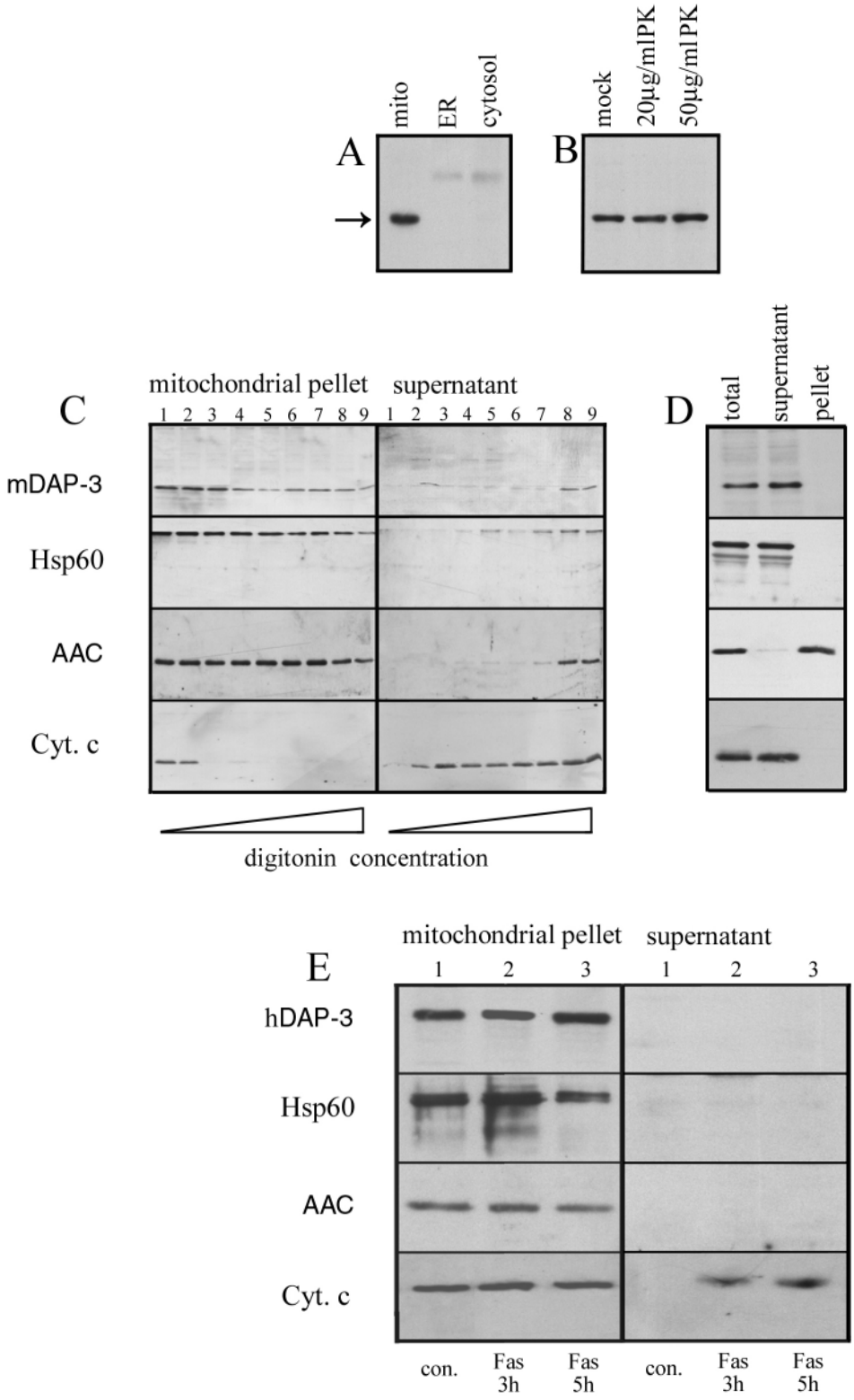

Fig. 5. Cell fractionation of rat liver homogenate. (A) Western blot with a hDAP-3 antibody of the different subcellular compartments: mitochondria (mito), endoplasmatic reticulum (ER), and cytosol (cytosol). The specific mDAP-3 signal (arrow) is only detectable in the mitochondrial fraction. (B) Western blot after proteinase K (PK) digestion of the mitochondrial fraction. The mitochondrial fraction was either mock treated or treated with 20 µg/ml or 50 µg/ml proteinase K, respectively. The mDAP-3 protein remains intact, indicating an intramitochondrial localization of mDAP-3. (C) Digitonin treatment of isolated mitochondria. Rat mitochondria were isolated and incubated with increasing digitonin concentrations, from 0% (lane 1), 0.075%, 0.1%, 0.125%, 0.137%, 0.15%, 0.2%, 0.3%, to 0.4% (lane 9) for 2 minutes on ice. After incubation mitochondria were pelleted, and supernatant and mitochondria were submitted to western blot analysis for mDAP-3, heat shock protein 60 (HSP60), ADP/ATP carrier (AAC) and cytochrome c (Cyt. c). mDAP-3 is released only at high digitonin concentrations as are HSP60 and AAC (supernatant lane 8 to 9), consistent with a localization in the mitochondrial inner membrane or matrix. Cytochrome c is already released at a very low digitonin concentration (0.1%; supernatant lane 3) consistent with its localization in the mitochondrial intermembrane space. (D) Carbonate treatment of isolated mitochondria. Isolated mitochondria were incubated in 0.1 M Na_2CO_3 for 30 minutes and split into two volumes. One volume was directly trichloracetate precipitated, representing the total proteins. The second volume was separated in pellet and supernatant. The pellet contains integral membrane proteins, the supernatant contains soluble or membrane associated proteins. Fractions were analyzed by western blot for mDAP-3, HSP60, AAC, and cytochrome c. mDAP-3 is found in the supernatant as are HSP60 and cytochrome c, indicating a soluble or membrane associated protein. AAC as an integral membrane protein is found in the pellet fraction. (E) Western blot of mitochondrial and cytosolic fractions of Jurkat cells stimulated to undergo apoptosis with an agonistic anti-Fas antibody and harvested at 3 hours (lane 2, 45% apoptotic cells) and 5 hours (lane 3, 54% apoptotic cells) after stimulation. hDAP-3 could only be detected in the mitochondrial fraction. In contrast, cytochrome c could be seen in the cytosol of cells undergoing apoptosis. lane 1, unstimulated control cells; lane 2, 3 hour Fas antibody stimulation; lane 3, 5 hour Fas antibody stimulation.

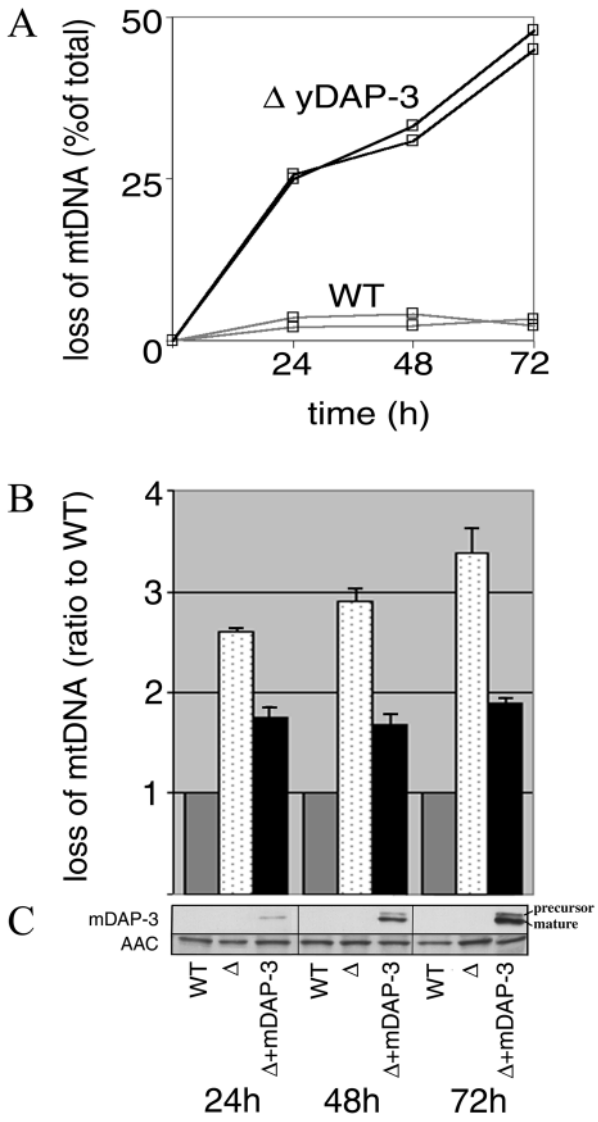

Fig. 6. Loss of mitochondrial DNA by the yDAP-3 null mutant and rescue with mDAP-3. (A) yDAP-3 disrupted yeast cells (ΔyDAP-3) were significantly defective in the maintenance of the mitochondrial genome compared to wild type (WT). (B) Transfection of the ΔyDAP-3 mutants (Δ) with mDAP-3 (Δ + mDAP-3) led to a partial reduction of the mitochondrial DNA loss. Experiments were performed in triplicates, ratio to wild type are given as median ± standard deviation. (C) mDAP-3 was efficiently processed to its mature size indicating import of the mammalian orthologue into yeast mitochondria and only expressed in transfected yeast cells. AAC is shown in the lower panel for control.

Amino acid sequence analysis of mDAP-3 with the program PSORT reveals a putative mitochondrial localization of mDAP-3. Similar results were obtained for hDAP-3 and yDAP-3. This subcellular localization of mDAP-3 to the mitochondria was further supported by three independent approaches. First, the mDAP-3/EGFP fusion protein showed a punctate mitochondrial pattern of mDAP-3 distribution by fluorescence microscopy. Second, the mDAP-3/EGFP fusion protein co-localized with a mitochondrial staining demonstrated by confocal microscopy. Third, liver cell fractionation analysis confirmed the mitochondrial localization of mDAP-3 biochemically. Furthermore, proteinase K and digitonin treatment of isolated mitochondria confirmed the intramitochondrial localization of mDAP-3. Carbonate treatment experiments showed, as expected from the sequence prediction, no integral association of mDAP-3 with mitochondrial membranes. Our data therefore localize mDAP-3 to the mitochondrial matrix. An association with the inner membrane via binding to integral membrane proteins remains possible. Sonication of isolated mitochondria, which ruptures the membranes, and subsequent western blot analysis of membrane- and soluble-fractions revealed an equal distribution of mDAP-3 between these two fractions (data not shown), suggesting an interaction of mDAP-3 with integral membrane proteins of the inner mitochondrial membrane.

The regulation of apoptosis by mitochondria is a matter of intense research (Alnemri, 1999; Martinou, 1999; Susin et al., 1998). Several evolutionary conserved mitochondrial proteins essential for oxidative phosphorylation play an additional proapoptotic role. These mitochondrial factors implicated in apoptotic function have been localized to either the outer or inner mitochondrial membrane, or the intermembrane space.

Cytochrome c, a highly conserved electron shuttle in the mitochondrial intermembrane space, was the first molecule identified to be a key player in caspase activation after release from mitochondria. Another electron carrier molecule, the flavoprotein AIF appears to be involved in apoptosis induction after translocation to the nucleus (Susin et al., 1999). These two phylogenetically old proteins play an intramitochondrial electron acceptor/donor function and a second, independent apoptogenic role in higher eukaryotes after release from mitochondria. DAP-3, in contrast to cytochrome c, retained its intramitochondrial localization during Fas induced cell death, indicating an intramitochondrial mode of action during apoptosis.

ANT-1, a highly expressed protein of the mitochondrial permeability transition complex, which is under physiological conditions a strictly specific ADP/ATP antiporter, can induce apoptosis after overexpression or by stimulation through a secondary signal (Bauer et al., 1999). This indicates an inhibition of ANT-1s proapoptotic function by other proteins of the permeability transition pore. For inhibition of apoptosis a delicate stoichometric balance between the various components of the transition pore appears to be required. A similar inhibition of the proapoptotic function of mDAP-3 by interacting molecules in non-apoptotic cells can be hypothesized, since mDAP-3 is also highly expressed in normal mitochondria.

As for cytochrome c the presence of DAP-3 homologues in non-apoptotic eukaryotes such as yeast indicates a function besides the regulation of apoptosis. The characterization of this function will be important to understand the molecular role for mitochondrial metabolism of this conserved protein family.

mDAP-3 showed significant amino acid identity to the yeast molecule YGL129c, here referred to as yDAP-3. This molecule contains the same conserved P-loop sequence as mDAP-3/hDAP-3/Apaf-1/CED-4. yDAP-3 mutants were reported to be unable to grow on glycerol as their sole carbon source (Tizon et al., 1999), indicating a yeast phenotype typically seen with disturbance of mitochondria (Lawson and Douglas, 1988).

However, our data indicate, that yDAP-3 is not directly required for respiration, but in the absence of yDAP-3 yeast cells tend to loose the mitochondrial genome. A direct role of yDAP-3 in mitochondrial DNA replication seems unlikely since the DNA loss was only moderate although significant. Loss of mitochondrial DNA was observed in yeast mutants defective in a variety of mitochondrial processes. For example mitochondrial morphology mutants often contain an unstable mitochondrial genome (Rapaport et al., 1998). However morphology of mitochondria in the yeast ΔDAP-3 strain did not show an altered morphology (data not shown). The molecular function of yDAP-3 in mitochondrial biogenesis seems to be conserved among the different members of the DAP-3 family, since the yeast mutant could be partially complemented by the mammalian mDAP-3 orthologue. Interestingly, the disruption of a yeast homologue to the ANT-1 transporter, RIM-2, also resulted in a loss of mitochondrial DNA (Van Dyck et al., 1995). These data might indicate a common, but so far unknown role of the ANT-1 and DAP-3 proteins for mitochondrial biogenesis and apoptosis induction. The yeast system should allow the identification of this function in the near future.

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