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A Conserved N-Terminal Sequence Targets Human DAP3 to Mitochondria

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Human DAP3 (death-associated protein-3) has been identified as an essential positive mediator of programmed cell death. Structure–function studies have shown previously the N-terminal extremity of the protein to be required in apoptosis induction. Analysis of human DAP3 gene structure predicted 13 exons and subsequent targeting prediction by two software packages (MITOPROT and TargetP) gave a high probability for mitochondrial targeting. The predicted N-terminal targeting structure was also found in the mouse, *Drosophila,* and *C. elegans* orthologues with a strong sequence homology between mouse and human. Secondary structure analyses identified α-helical structures typical of mitochondrial target peptides. To confirm experimentally this targeting we constructed a fusion protein with N-terminal human DAP3 upstream of enhanced green fluorescent protein (EGFP). Confocal analysis of transfected human fibroblasts clearly demonstrated EGFP localization exclusive to mitochondria. The positioning of this key apoptotic factor at the heart of the mitochondrial pathway provides exciting insight into its role in programmed cell death.

Key Words: **DAP3; mitochondria; targeting; apoptosis.**

Death-associated protein-3 (DAP3) is one of five major positive mediators of apoptosis identified by Kissil *et al.* (1995) using Technical Knockout, a technique based on random inactivation of mRNAs with an antisense cDNA library and positive selection of cells surviving in the presence of an apoptotic stimulus (1). The human DAP3 gene has been localized to chromosome 1q21 (2); however, the precise gene structure of the human protein has not yet been reported. The gene is transcribed into a 1.7-kb mRNA and tissue expression is ubiquitous (1). The corresponding protein of 46 kDa has been localized by immunostaining to cytoplasmic vesicles (3). The human DAP3 protein has no known homologues and is evolutionary highly conserved. For example the *C. elegans* protein shares 35% identity and 64% similarity with the human protein (4). Structure–function studies of DAP3 have demonstrated the essential domains of this protein. Mutation of a conserved P-loop motif significantly reduced DAP3 induced cell death, suggesting the essential role of a putative nucleotide binding activity (4). Deletion studies have identified that truncation of minimal regions comprising 68 amino acids from the C-terminus or 100 amino acids from the N-terminus (exclusive of the P-loop) was sufficient to abolish completely the death promoting action of DAP3. In contrast, the N-terminal 230 amino acids act in a dominant-negative fashion with this activity dependent on the presence of a functional nucleotide binding motif (4).

Overexpression of DAP3 leads to a significant increase in cell death but its precise function within the apoptotic pathway is at present undefined. DAP3 appears to act downstream of pro-apoptotic stimuli such as interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) and Fas ligand and upstream to several caspases, other than caspase 8 (1, 4). It has also recently been shown that human DAP3 binds in a ligand-dependent manner several members of the nuclear receptor family such as the glucocorticoid receptor and thyroid hormone receptor (5).

We found DAP3 to be overexpressed in thyroid oncocytoma using differential expression studies (unpublished data). Oncocytic cells are principally characterized by an accumulation of abnormal mitochondria (6). This finding led us to investigate the possible mitochondrial localization of DAP3. We here describe the bioinformatics and experimental data, which demonstrate the mitochondrial targeting of human DAP3.

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FIG. 1. Target peptide structure of human DAP3 and comparison with other species. (A) Sequence homology between human and mouse DAP3 is indicated by shaded letters and the predicted α-helical structures are illustrated by underlining. Arrows indicate the position of putative cleavage sites. (B) Sequence homology between *Drosophila* and *C. elegans* DAP3 is indicated by shaded letters and predicted α-helices are illustrated by underlining. Arrows indicate the position of putative cleavage sites.

TABLE 1

Prediction of Mitochondrial Targeting of DAP3

Species	MitoProt: Probability	TargetP	
		Score	Reliability class
Homo sapiens	0.8964	0.869	3
Mus musculus	0.7593	0.813	3
Drosophila m.	0.9852	0.867	2
C. elegans	0.9927	0.938	1

Note. The reliability classes of TargetP decrease from a score of 1 (97% specificity) to 5 (53% specificity).

EXPERIMENTAL PROCEDURES

Sequence analysis of human DAP3. To analyze gene structure the human cDNA sequence of DAP3 was submitted with BLASTN against the "htgs" database (Unfinished High Throughput Genomic Sequences). The human cDNA exon/intron structure of DAP3 was determined, although the precise order of the five corresponding contigs was not complete (clone: AL162734).

Computer targeting and α-helices prediction. Targeting prediction was performed using two different software packages, TargetP (http//www.cbs.dtu.dk/services/TargetP/) and MITOPROT (http://websrv.mips.biochem.mpg.de/proj/medgen/mitop/). Claros and Vincens (7) described MITOPROT in 1996 and TargetP was developed by Emanuelsson *et al.* (2000) (8). MITOPROT was used for prediction of both targeting and putative cleavage sites. Prediction of α-helical structures was performed using the secondary structure program "Predator" at the Predict Protein server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html). Protein sequences were extracted from Swissprot and SPTREmbl databases, human DAP3: Swiss Prot P51398, mouse: SPTREmbl CAC05583, *Drosophila:* SPTREmbl Q9W253, *C. elegans:* SPTREmbl Q9TVM5.

Plasmid Constructs. The fusion protein N-termDAP3-EGFP was constructed as follows. Specific primers were used to amplify a 441 base pair 5′ fragment of human DAP3 cDNA, and to introduce a *Bgl*II restriction site by PCR mutagenesis (DBglII: CCTAGGCTGAGA**T**CTAGTC and RHindIII: GATATCGTATAGCTGGATAAGC). The resultant PCR product was sub-cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). A *Bgl*II/*Hind*III 314-base pair, coding for amino acids 1–96, was then cloned in frame into appropriately digested EGFP-N3 vector (Clontech). The junction of N-terminal DAP3 and EGFP in the constructed plasmid was verified by sequencing. The N-terminal DAP3 fragment was subsequently expressed as an N-terminal fusion protein of EGFP. EGFP-N3 alone was used as a negative control of targeting.

Cell culture and transient transfections. The human diploid fibroblast cell line WI38 was cultured in Dulbecco's modified Eagle medium + F12 (Gibco BRL) containing pyridoxine and 15 mM Hepes supplemented with 10% (v/v) fetal-bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded on coverslips in a 6 well plate at a density of 5×10^5 cells per well and cultured for 24 h. Transient transfections were performed with Lipofectamine transfection reagent (Gibco BRL) according to the manufacturer's protocol. Cells were incubated with 1 μg DNA per well for 6 h in the presence of serum-free media, Optimem (Gibco BRL), and a further 18 h in the presence of serum. Fusion protein expression was examined 48 h posttransfection.

Confocal microscopy. WI38 cells were grown on coverslips and transfected with different constructs as described. Mitochondrial staining was performed by incubating cells with Mitotracker red CMXRos (Molecular Probes) (20 nM) for 15 min at 37°C. Cells were subsequently washed twice in PBS, fixed for 10 min in 4% paraformaldehyde and mounted with Vectorshield (Vector Laboratories). Cells were analyzed on a Leica DMIRBE laser-scanning microscope equipped with a 100× oil immersion objective. The 488-nm laser was used for excitation of EGFP and the 568 laser for excitation of Mitotracker red. Initial image analysis was performed using Leica TCS NT software and subsequently images were exported to Adobe Photoshop (Adobe Systems Inc.).

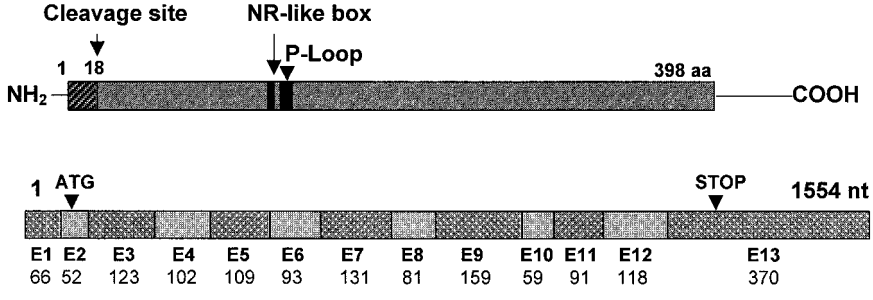

FIG. 2. Exon/intron structure of the predicted human DAP3 gene. Exon size is given in base pairs. Arrows indicate the P-loop, a nuclear receptor-interacting domain (NR box)-like motif and putative cleavage site of the target peptide.

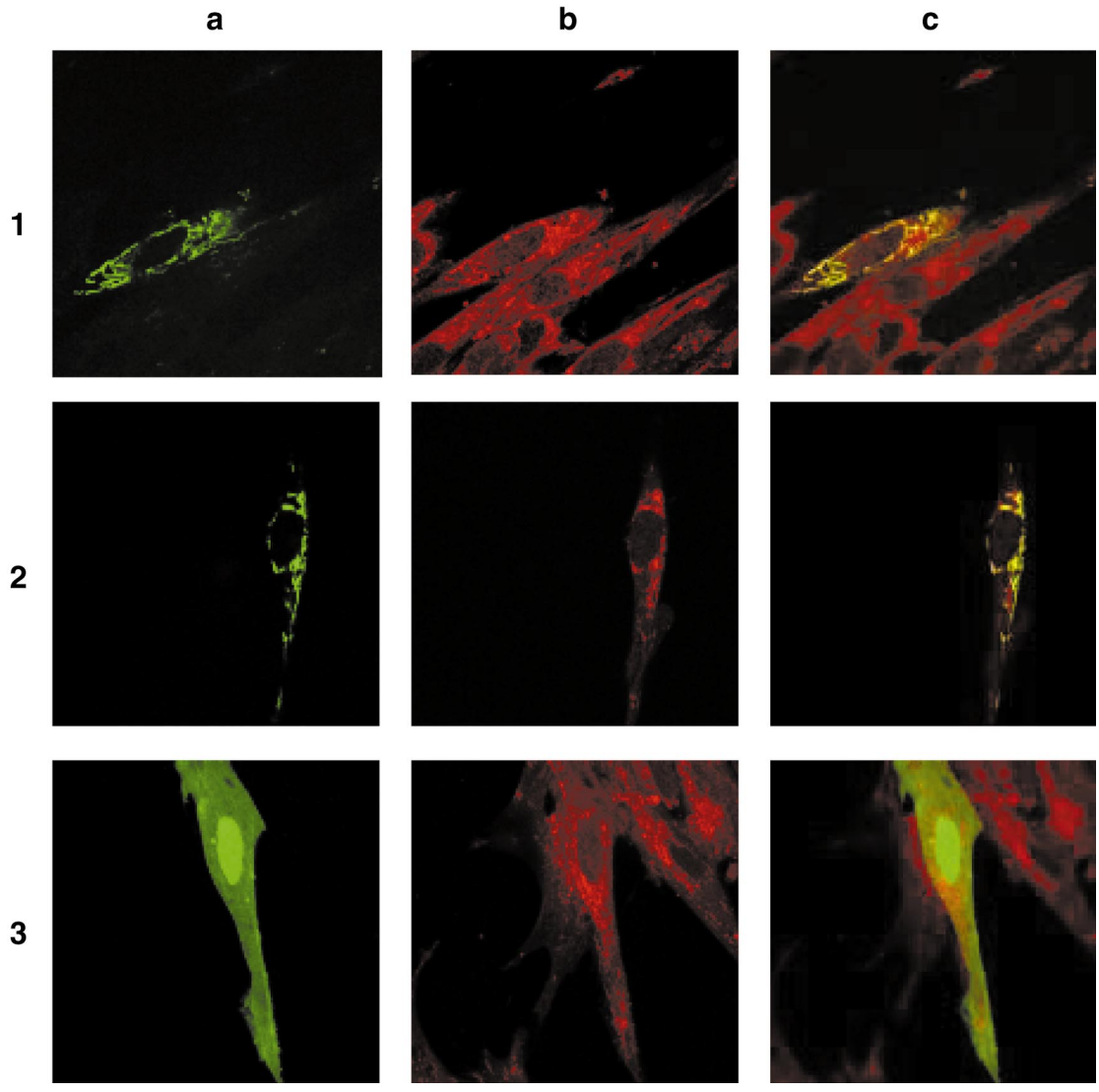

FIG. 3. Localization of the N-terminal fusion protein NtermDAP3-EGFP to mitochondria. (a) EGFP fluorescence, (b) Mitotracker red fluorescence, and (c) the overlaid images of a and b. Panels 1 and 2 illustrate cells transfected with the fusion protein NtermDAP3-EGFP and panel 3 transfection with the negative control, EGFP alone. In c1 and c2 the specific targeting of N-termDAP3-EGFP is demonstrated by the colocalization of red and green fluorescence.

RESULTS AND DISCUSSION

Mitochondrial import sequences are often cleavable N-terminal peptides, extremely variable in length and sequence yet characterized by several physicochemical properties (7–9). For instance, the presequence is enriched in Arg, Leu, Ser and Ala, but has a reduced content of acidic residues. It contains a minimum of two positively charged amino acids and has the potential to form amphiphilic α-helical structures (7–9). Using two prediction software packages we showed high probability scores for mitochondrial targeting of human DAP3 (Table 1). The four species studied displayed a high score with *C. elegans* demonstrating the best prediction. Secondary structure analysis predicted a typical α-helix in the N-terminus of each species studied. The characteristic physicochemical N-terminal structure was found in all four species and between human and mouse there was also a strong sequence homology as illustrated in Fig. 1. N-terminal mitochondrial import sequences are frequently cleaved from the pre-protein within the organelle (10, 11). The MITOP package predicted a putative cleavage site at amino acid 18 of human DAP3 and at amino acid 17 in the mouse orthologue (Fig. 1). This conservation of mitochondrial targeting strongly suggests that mitochondrial localization of DAP3 is essential for the function of the protein. In contrast gene structure of DAP3 is not conserved. We found the

human gene to contain 13 exons (Fig. 2) distributed over about 60 kb while the *C. elegans* gene was reported to contain just 7 exons (4).

To confirm experimentally the predicted targeting of human DAP3 to mitochondria we constructed an N-terminal fusion protein. The first 96 amino acids of the human DAP3, containing the putative targeting sequence, were cloned upstream of enhanced green fluorescent protein (EGFP). Cells transfected with either EGFP-N3 alone or NtermDAP3-EGFP were analyzed by confocal microscopy. NtermDAP3-EGFP fluorescence was localized exclusively to mitochondria as shown by colocalization with the mitochondrial marker Mitotracker red (Fig. 3). This contrasted dramatically with the homogenous labeling of cells transfected with EGFP alone, where fluorescence was observed throughout the cytoplasm and nucleus. These results demonstrate human DAP3 to be exclusively targeted to mitochondria. In support of these findings mitochondrial localization of the mouse orthologue has also just been shown by transfection studies using a fusion protein containing the whole mDAP3 cDNA sequence cloned upstream of the EGFP reporter gene (12). Our results also demonstrate that targeting is mediated by the N-terminal extremity of the protein, as is the case for many mitochondrially targeted proteins (7–9).

Human DAP3 has previously been shown to act downstream of several apoptotic stimuli (TNFα, IFNγ and FAS ligand) and caspase 8 (1, 4). The mitochondrial localization of this essential pro-apoptotic protein is therefore not surprising, as TNFα is well known to mediate apoptosis through mitochondrial pathways (13, 14). Although Fas ligand-mediated apoptosis can short-circuit the mitochondrion and activate directly the downstream caspases, it has also been shown to stimulate Bid translocation to the mitochondrion and subsequent cytochrome *c* release (13–15). Mitochondrial targeting of human DAP3 could be important for its pro-apoptotic effects since deletion of amino acids 1–100 from the N-terminus is sufficient to abrogate this function (4).

Recent work has presented interesting new data on human DAP3 function by demonstrating the ability of this protein to associate *in vitro* with several nuclear receptors (glucocorticoid receptor, thyroid hormone receptor, and peroxisome proliferator-activator receptor), some basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) proteins and heat-shock protein 90 (hsp90) (5). Association with nuclear receptors is mediated primarily by a nuclear receptor-interacting domain (NR box)-like motif, which mapped to the N-terminus in close proximity to the P-loop motif. In contrast, DAP3 interactions with bHLH/PAS proteins and hsp90 are probably mediated by a C-terminal motif, which resembles the PAS B domain sequence. Co-transfection studies showed DAP3 able to enhance steroid sensitivity and to induce a 10-fold increase in ligand-induced transcriptional activation by glucocorticoid receptor.

In the light of our current study demonstrating the mitochondrial localization of DAP3 its association with nuclear receptors is difficult to understand. It is possible that a small fraction of DAP3 undetectable by fluorescence may not be targeted to the mitochondria and thus would be available for interaction with nuclear receptors. Alternatively, this ambiguity could be explained by the presence of "nuclear" receptors within mitochondria. Glucocorticoid and truncated thyroid hormone receptors as well as a PPARγ2-like protein have been localized to mitochondria and suggested to stimulate mitochondrial DNA transcription (16–20), but their precise mode of action is still unclear. This presence of "nuclear" receptors in the mitochondrion would however resolve the "geographical" dilemma. Interestingly, recent findings demonstrated that the translocation of the nuclear orphan receptor TR3 to mitochondria can induce apoptosis independently of its transcriptional activity (21). Further studies are required to investigate whether the nuclear binding activity of DAP3 is linked to its pro-apoptotic actions. Nevertheless, it is clear that DAP3 acts as an essential mediator of programmed cell death and the demonstration of its mitochondrial localization, in mouse and human, provides promising new avenues of research.

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