

In vivo localisation and stability of human Mcl-1 using green fluorescent protein (GFP) fusion proteins

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Abstract Mcl-1 is an anti-apoptotic member of the Bcl-2 family of proteins. We have expressed full length and mutated GFP:Mcl-1 fusion proteins to define structural motifs that control protein localisation and stability. When expressed in U-937 cells, full length Mcl-1 locates primarily within mitochondria and its half-life was approximately 3 h, which was identical to the native, endogenously expressed protein. When the terminal 20 amino acids from the C-terminus of the protein were detected, the protein was diffused in the cytoplasm, but its stability was unaffected. This confirms that this region is responsible for efficient targeting to mitochondria. Surprisingly, deletion of 104 amino acids (residues 79–183) that contain putative PEST sequences and other stability regulating motifs, did not affect protein stability. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: U-937; Apoptosis; Bcl-2; Fluorescence imaging; Mitochondrion

1. Introduction

The Bcl-2 family of proteins plays a central role in the regulation of apoptosis in various cells and tissues [1–3]. Some members of this family protect against apoptosis (such as Bcl-2 itself, Bcl-X_L, A1(Bfl-1) and Mcl-1) whereas others promote apoptosis (e.g. Bax, Bcl-X_S, Bad, Bid, Bim and Bak). The functions of these molecules appear to be determined by conserved amino acid sequences within the proteins that are termed BH (Bcl-2 homology) domains [4,5]. The anti-apoptotic family members contain three or four BH domains (BH1–4) with the BH4 domain found in most anti-apoptotic family members with the exception of Mcl-1 and A1 [6–9]. This BH4 domain is reported to be essential for the anti-apoptotic actions of Bcl-2 and Bcl-X_L. Some pro-apoptotic family members possess three domains, but some, the so-called ‘BH3-only’ proteins contain only one of these (BH3), indicating that this may be an essential death-promoting domain [10,11]. In the anti-apoptotic proteins, BH1–3 form a hydrophobic cleft and it may be that BH3 only proteins bind this cleft [12].

Mcl-1 was originally cloned as an early induction gene from the haematopoietic cell line ML-1, following induction of differentiation by phorbol ester [13]. Analysis of the predicted amino acid sequences encoded by this cDNA identified BH1–3 domains and a putative hydrophobic transmembrane (TM)

domain likely to be important in membrane anchoring of the protein (Fig. 1A). There is good evidence to show that Mcl-1, like Bcl-2 and Bcl-X_L, can function as a survival protein as its over-expression following transfection confers temporary resistance to apoptosis in cell lines or in transgenic mice [14–16]. However, Mcl-1 (40/42 kDa) is a much larger protein than Bcl-2 (26 kDa) or Bcl-X_L (29 kDa), mainly due to the presence of amino acid stretches towards the N-terminal region of the protein, than are believed to be important for its unique properties [17]. The N-terminal region of the protein contains PEST sequences and Arg:Arg motifs that are commonly found in proteins with a high rate of turnover [18]. Thus, Mcl-1 is predicted to have a high turnover rate [13,19]. Furthermore, the carboxy region of the protein is rich in hydrophobic amino acids and this region is predicted to serve as a transmembrane anchor. This region is again different to that of Bcl-2 implying that the proteins may insert into different membranes. In addition to its high turnover rate, Mcl-1 expression can be rapidly induced in a variety of cell types (e.g. [13,20–22], indicating that cellular levels can be rapidly up- or down-regulated. Thus whilst Mcl-1 can perform a similar function to Bcl-2 in protection from apoptosis, its expression is independently regulated and it may be more important than Bcl-2 in certain cells and tissues at different stages in their differentiation programme [23]. For example, Mcl-1 is rapidly and transiently induced (whilst Bcl-2 levels decrease) in myeloid cells during DNA damage [24], and during B cell development Mcl-1 expression is transiently induced prior to induction of Bcl-2 expression [25]. Human neutrophils do not express Bcl-2 (nor Bcl-X_L), but do express Mcl-1, and levels of expression of this protein correlate with survival of these cells in response to agents such as GM-CSF and LPS [21]. Antisense disruption of Mcl-1 induces apoptosis in PMA-treated U-937 cells [26] and in human neutrophils (unpublished observation). A recently reported alternatively spliced variant of Mcl-1 that has lost its BH1 and BH2 domains to become a ‘BH3-only’ molecule, can induce apoptosis [27].

Previous work using immuno-cytochemistry has localised Mcl-1 protein to light intracellular membranes, including mitochondria [19], and there is some evidence to suggest that even in cells co-expressing Bcl-2 and Mcl-1, these two proteins may be located at different sub-cellular sites [28]. In the present studies we have expressed Mcl-1 as green fluorescent protein (GFP) fusion proteins in order to determine its sub-cellular localisation by non-invasive fluorescence imaging. We have also expressed mutant forms of GFP:Mcl-1 fusion proteins that have deleted TM domains and deleted PEST sequences and determined the effects of these mutations on protein targeting and stability. Full length Mcl-1 was localised to mitochondria whereas a mutant lacking the TM domain was

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located diffusely throughout the cytoplasm. Surprisingly, a mutant with deleted PEST sequences had the same short half life (approximately 3 h) as native Mcl-1. Thus, other, as yet unidentifiable, factors contribute to the instability of this highly labile protein.

2. Materials and methods

2.1. Materials

pEGFP-C3 Vector and anti-GFP peptide antibodies were from Clontech. pBluescript KS Vector and XL1-Blue cells were obtained from Stratagene Ltd. Mcl-1 antiserum was from Pharmingen. Anti-rabbit IgG-HRP secondary antibody, ECL detection reagent and Hyperfilm ECL were all from Amersham. All restriction, amplification, modification enzymes and the High Pure PCR Product Purification kit were from Boehringer Mannheim. RPMI 1640 was from ICN Biomedicals Ltd, foetal calf serum (FCS) was from Gibco BRL. Mitotracker Red CMXRos was from Molecular Probes Europe B.V. All other specialist materials were from Sigma. Oligonucleotide primers were synthesised by MWG-Biotech Ltd.

2.2. Cell lines and cell culture

The U-937 human monocytic cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured at 37°C under 5% CO₂ in RPMI 1640 supplemented with 10% FCS and 1 mM L-glutamine. Cells were fed 16–20 h prior to the transfection experiments.

2.3. Reverse transcription and PCR amplification of the coding region of the human mcl-1 gene

Human *mcl-1* cDNA encompassing the entire coding region (~1100 bp) was synthesised by reverse transcription and PCR amplification of total RNA isolated from 3 h PMA-stimulated U-937 cells using two gene specific primers [22]. A sense primer, containing a *Hind*III site at its 5'-end (5'-GATAAGCTTCGACTGGCAATGTTTGGC-3') and an antisense primer, containing a *Bam*HI site at its 5'-end (5'-AATGGATCCTCTTGCCACTTGCTTTTCTG-3') were used. 5 µg total RNA was reverse transcribed with 2.5 µg antisense primer. The cDNA was then amplified by 35 cycles of PCR (denaturing temperature 94°C, annealing temperature 54°C, amplification at 72°C). 500 ng of the pEGFP-C3 vector and PCR product were restricted by *Hind*III/*Bam*HI. The restricted vector and the PCR products were gel-purified, ligated and cloned. The cDNA insert was sequenced to verify its identity and absence of mutation. pEGFP-C3(Mcl-1) was used for transfection and localisation of the fusion protein. Fusion of Mcl-1 to the C-terminus of GFP ensured that the putative TM domain of Mcl-1 was free to insert into its target membrane(s).

2.4. Creation of pEGFP-C3(Mcl-1[ΔTM]) and pEGFP-C3(Mcl-1[ΔPEST]) clones

pEGFP-C3(Mcl-1[ΔTM]) was prepared by deleting the TM domain (Fig. 1A) from pEGFP-C3(Mcl-1). A sense primer, was used for amplification of the coding region, (5'-GATAAGCTTCGACTGGCAATGTTTGGC-3') and an antisense primer which by-passed the TM domain, creating a new stop codon and containing a *Bam*HI site at its 5'-end (5'-CTGATGGATCCTTATAGGTCCTCTAC-3') was used to amplify the coding region minus the TM domain [13,22]. The PCR product was cloned into pEGFP-C3 between the *Hind*III and *Bam*HI sites. pEGFP-C3(Mcl-1[ΔTM]) was used for transfection and localisation of the fusion protein. pEGFP-C3(Mcl-1[ΔPEST]) was prepared by deleting a region containing PEST motifs (Fig. 1A). Briefly, the pEGFP-C3(Mcl-1) was restricted with *Not*I/*Asp*718 and cohesive ends produced were blunt ended. The blunt-ended fragment was gel-purified and self-ligated. These steps created a new clone, pEGFP-C3(Mcl-1[ΔPEST]) which did not contain the PEST motifs and did not change the reading frame of the downstream coding region. Successful amplification of both products was confirmed by sequencing.

2.5. Transient transfection of the fusion protein constructs

Logarithmically growing U-937 cells were resuspended in RPMI 1640 supplemented with 10% FCS and 1 mM L-glutamine at a concentration of 1×10^7 cells/800 µl and at least 40 µg of each construct

DNA was added. Cells were electroporated with a single pulse from a Bio-Rad gene pulser apparatus with a capacitance extender unit (250 V, 960 µF). Transfected cells were incubated on ice for at least 10 min before and after transfection, and then resuspended in 20 ml of RPMI 1640 supplemented with 10% FCS and 1 mM L-glutamine. Transfected cells were incubated in 24-well plates.

2.6. Western blotting

Following transfection, cells were incubated for 40 h at 37°C in 5% CO₂. The cells were then treated with 10 µg/ml cycloheximide to inhibit protein synthesis and incubated for the indicated times. Whole cell extracts were prepared by resuspending 6×10^5 cell aliquots each in 100 µl SDS-PAGE reducing sample buffer and boiled for 5 min. These extracts were separated by SDS-PAGE using 10% acrylamide with 2×10^5 cell equivalents loaded per lane. Following electrophoresis, proteins were transferred to PVDF membranes using a Bio-Rad Mini Protean II Transfer apparatus. The membranes were incubated with primary antibodies (anti-Mcl-1 primary antibody (1:2000) and (1 µg/ml) anti-GFP peptide antibody) overnight at 4°C. The membranes were then washed for 2×30 s and 3×5 min in wash buffer

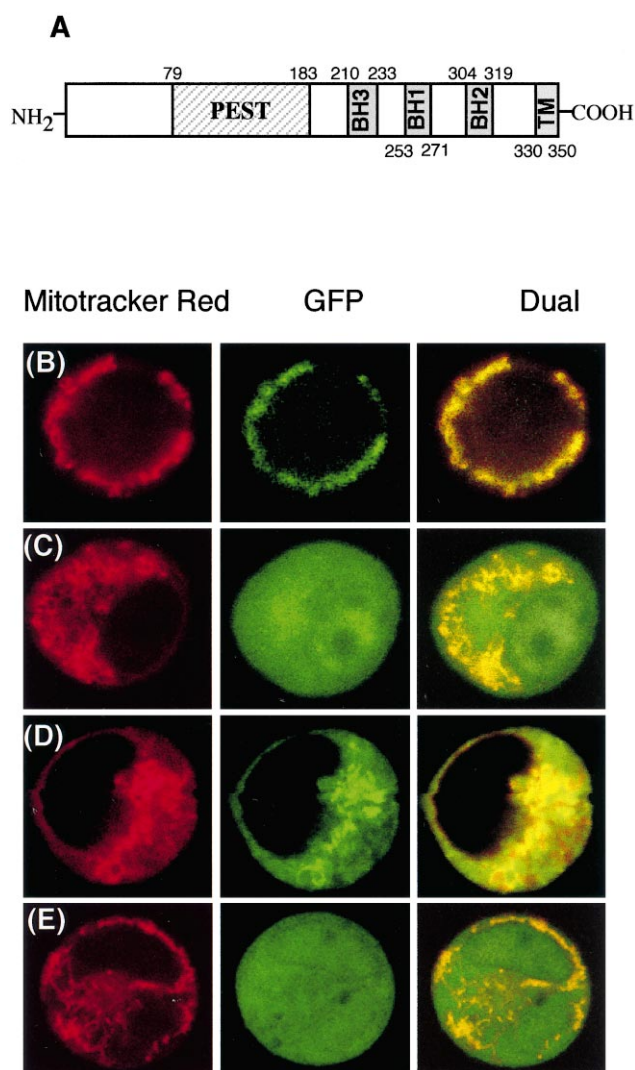


Fig. 1. Subcellular localisation of GFP:Mcl-1 fusion proteins. A: Shows the structure of the Mcl-1 protein with putative PEST regions, BH domains and putative TM domains. Numbers refer to amino acid residues. U-937 cells were transfected with plasmids encoding (B) full length GFP:Mcl-1; (C) GFP:Mcl-1 (ΔTM); (D) GFP:Mcl-1 (ΔPEST) or (E) GFP alone. After 40 h incubation cells were stained for 30 min with 20 ng/ml Mitotracker Red CMXRos and viewed for red and green fluorescence by confocal microscopy.

before incubation with HRP-linked rabbit secondary antibody for 1 h at room temperature. Signals were developed using Amersham's ECL detection reagent. Images were captured by using a Hamamatsu XC-77CE CCD camera and processed using Image 1.44 VDM software.

2.7. Confocal microscopy

After 40 h incubation, transfected cells were prepared for microscopy by incubation with 20 ng/ml of a mitochondria-specific dye, Mitotracker Red CMXRos, for 30 min in cell culture medium. After mitochondrial staining the cells were washed twice with and then suspended in PBS pH 7.4. Resuspended cells were placed on microscope slide and images were collected on an LSM 510 confocal microscope. The 488- and 568-nm lines of krypton-argon laser were used for fluorescent excitation of GFP and Mitotracker Red CMXRos, respectively.

3. Results and discussion

3.1. Localisation of full length and mutant GFP:Mcl-1 fusion proteins

Expression of full length Mcl-1 fused to the C-terminus of GFP, was predicted not to affect the TM domain of the pro-

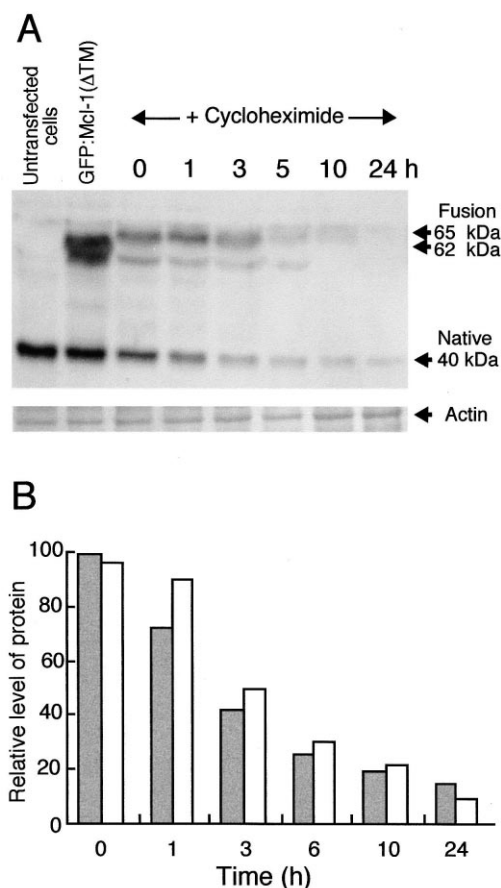


Fig. 2. Expression and stability of full length GFP:Mcl-1 fusion proteins. U-937 cells were transfected with a plasmid encoding a full length GFP:Mcl-1 fusion protein. After 40 h incubation, cells were incubated with 10 μ g/ml cycloheximide to inhibit protein synthesis and then incubated for a further 24 h. At time intervals, aliquots were removed for immuno-blot analysis using anti-Mcl-1 antibodies. After immuno-staining, filters were stained with Ponceau red for visualisation of the actin signal. A: Shows immuno-blot analyses whilst B shows quantitation of the immuno-blot signals of both the GFP:Mcl-1 fusion protein (65 kDa) and native, endogenously expressed Mcl-1 protein (40 kDa). Representative results from three separate experiments.

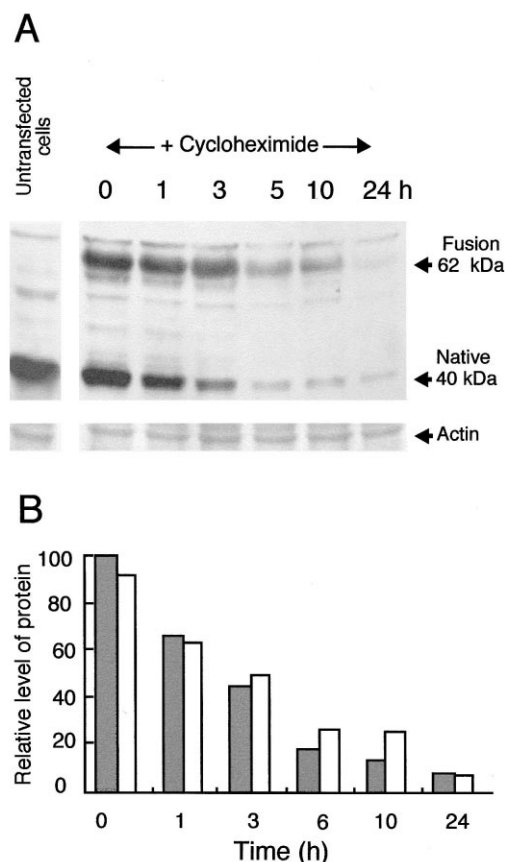


Fig. 3. Expression and stability of GFP:Mcl-1 (Δ TM) fusion proteins. Experimental details are as in the legend to Fig. 2 except that cells were transfected with a plasmid encoding GFP:Mcl-1 (Δ TM). Representative result from three separate experiments.

tein and hence its membrane-binding properties. Mitochondria of U-937 cells stained with Mitotracker as red structures threaded through the cytoplasm. Full length GFP:Mcl-1 fusion protein clearly co-localised with these mitochondria (Fig. 1B). Similarly, GFP:Mcl-1 (Δ PEST) fusion protein also co-localised with mitochondria, indicating that these deleted regions of the protein do not play any role in protein targeting to membranes (Fig. 1C). However, GFP:Mcl-1 (Δ TM) fusion protein did not localise with mitochondria and clearly displayed diffuse cytoplasmic staining (Fig. 1D) as did native GFP (Fig. 1E). These experiments thus confirm that (a) full length Mcl-1 protein localises primarily to mitochondria and (b) the putative TM domain identified by consensus structural motifs in the predicted amino acid sequence of the cDNA [13] is genuinely responsible for protein targeting to these mitochondria.

3.2. Stability of native and GFP fusion proteins

U-937 cells transfected to express full length GFP:Mcl-1 fusion protein were incubated for 40 h and then incubated with 10 μ g/ml cycloheximide to inhibit de novo protein biosynthesis. At timed intervals samples were removed for analysis of Mcl-1 protein levels (both GFP:Mcl-1 fusion protein and native, endogenously expressed Mcl-1 protein) by immunoblotting. Fig. 2A shows that the full length GFP:Mcl-1 fusion protein ran as a major 65-kDa band, corresponding to the predicted size of the fusion protein. Also seen in Fig.

2 is endogenously expressed Mcl-1 running at approximately 40 kDa. After addition of cycloheximide, levels of both the Mcl-1 fusion protein and native Mcl-1 were seen to rapidly decline such that by 24-h incubation, only very weak signals were detected by immuno-blotting. This contrasts the levels of actin protein, which were relatively constant for the duration of the experiment. Densitometric analysis of these immuno-blot revealed that the half lives of both the full length GFP:Mcl-1 fusion protein and native Mcl-1 were virtually identical, at around 3 h (Fig. 2B). These experiments thus confirm the predicted short half life of both the expressed and native proteins.

Immunoblotting revealed that the major band seen in cells expressing the GFP:Mcl-1 (Δ TM) fusion protein was 62 kDa, confirming that the protein was approximately 3 kDa smaller than the full length protein due to the deletion of its putative TM domain (Fig. 3A). The half life of this protein was again remarkably similar to that of the native Mcl-1 protein, being approximately 3 h (Fig. 3B). Thus, whilst this mutated protein does not target efficiently to mitochondria (Fig. 1B), this lack

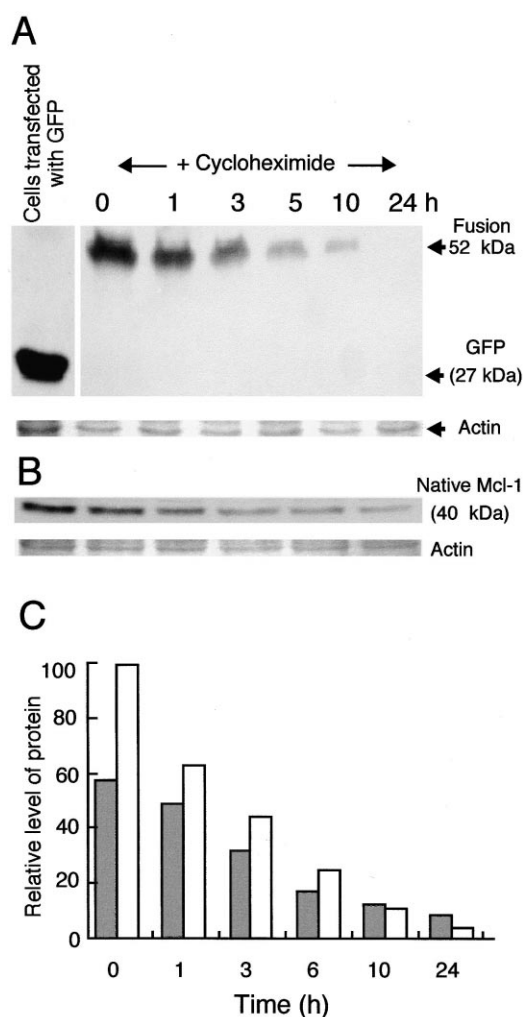


Fig. 4. Expression and stability of GFP:Mcl-1 (Δ PEST) fusion. Experimental details are as described in the legend to Fig. 2 except that cells were transfected with a plasmid encoding GFP:Mcl-1 (Δ PEST). In A, filters were blotted with an anti-GFP antibody, whilst in B, filters were probed with an anti-Mcl-1 antibody. C: Quantitation of the immuno-blot signals. Representative result from three separate experiments.

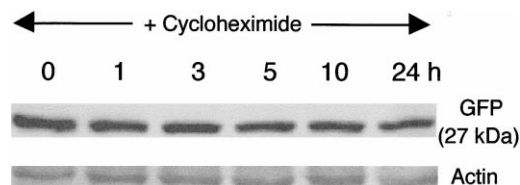


Fig. 5. Expression and stability of GFP. Experimental details are as described in the legend to Fig. 2 except that cells were transfected with a plasmid encoding GFP and filters were probed with an anti-GFP antibody.

of targeting neither increases nor decreases its stability. Therefore, the stability of Mcl-1 does not appear to be affected by its location within the cell. The expressed GFP:Mcl-1 (Δ PEST) fusion protein ran as a band of approximately 54 kDa (close to the predicted size of this mutant protein) and this protein was detected by immunoblotting using an antibody raised against GFP (Fig. 4A). This detection system was necessary because the antibody used to detect Mcl-1 was raised against a peptide corresponding to a region contained within this deleted PEST region of the mutant protein. Cells expressing native GFP protein showed a major band of approximately 27 kDa, corresponding to the known size of this protein. Remarkably, the half life of this GFP:Mcl-1 (Δ PEST) fusion protein was identical to that of either full length GFP:Mcl-1 or native Mcl-1, being approximately 3 h (Fig. 4B). In order to exclude the possibility that all proteins derived from this expression system had equally low stabilities, native GFP was expressed and its stability assessed. Fig. 5 shows that the stability of GFP in this experimental system was >24 h, indicating that the results seen in Figs. 2–4 were likely to be due to the inherent biological properties of the full length and mutant proteins.

In conclusion, we have shown that Mcl-1 protein is located mainly within the mitochondria of U-937 cells. The approach that we have used to reach this conclusion is based on expression of GFP fusion proteins, which has numerous advantages over techniques such as immuno-cytochemistry which involve fixation and permeabilisation of cells. Such techniques can often lead to artefacts in localisation due to non-specific antibody staining and/or redistribution of proteins during the fixation/permeabilisation procedures. We have also confirmed that the C-terminal domain of approximately 20 amino acids is indeed responsible for targeting the protein to mitochondria. However, deletion of some 104 amino acids containing putative PEST sequences does not increase the normally short half life of this protein. Clearly, factors outside of this region of the protein also play important roles in determining the stability of this short-lived protein.

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