The human homologue of the yeast mitochondrial AAA metalloprotease Yme1p complements a yeast *yme1* disruptant

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Abstract In yeast, three AAA superfamily metalloproteases (Yme1p, Afg3p and Rca1p) are localized to the mitochondrial inner membrane where they perform roles in the assembly and turnover of the respiratory chain complexes. We have investigated the function of the proposed human orthologue of yeast Yme1p, encoded by the *YME1L* gene on chromosome 10p. Transfection of both HEK-293EBNA and yeast cells with a green fluorescent protein-tagged YME1L cDNA confirmed mitochondrial targeting. When expressed in a *yme1* disruptant yeast strain, *YME1L* restored growth on glycerol at 37°C. We propose that *YME1L* plays a phylogenetically conserved role in mitochondrial protein metabolism and could be involved in mitochondrial pathologies.

Key words: Metalloprotease; AAA protein; ATPase; Yeast; Mitochondrion; *YME1*

1. Introduction

Members of the AAA superfamily of proteins in yeast are involved in mitochondrial protein metabolism, specifically in the incorporation of mitochondrial translocation products into the respiratory chain complexes and the turnover of mistranslated polypeptides [1]. Three mitochondrial metalloproteases of this superfamily have been characterized, that play an important role in mitochondrial biogenesis: Afg3p [2], Rca1p [3] and Yme1p [4]. Mutation or deletion of any of these genes results in phenotypic defects in respiration [1,5]. Inactivation of *YME1* has more general effects on mitochondrial integrity [6] and results in extremely slow growth of strains lacking mitochondrial DNA [4,7]. Conversely, expression of *Saccharomyces YME1* in *Schizosaccharomyces pombe* converts it to petite-positive [8].

YME1 appears to be required both for the degradation of unassembled subunits of cytochrome *c* oxidase, notably subunit 2 [9,10] and, based on in vitro experiments, for efficient assembly of the mitochondrial respiratory chain, via its chaperone-like activity [11,12]. A *yme1* disruptant strain is unable to grow on non-fermentable carbon sources at 37°C [4,13]. The gene name (yeast mitochondrial escape) is based on the phenotype first noticed in (null) mutants, namely an increased rate of mitochondrial DNA escape to the nucleus [4,14]. Multiple pathways for this process have been suggested [6,15], but the exact role of *YME1* in preventing it remains unclear.

Mitochondrial AAA proteins may have similar roles in humans to those in yeast, based on the molecular cloning of one member of the family, paraplegin [16], which shows greatest similarity to yeast Afg3p and Rca1p. Null mutations in the gene that encodes it (*SPG7*) cause one form of hereditary spastic paraplegia [16], and are associated with mitochondrial OXPHOS defects in patients. However, despite its similarity to the yeast metalloproteases, paraplegin was unable to complement any of three yeast strains disrupted, respectively, for *AFG3*, *RCA1* or *YME1* (G.A.J. Hakkaart, unpublished data).

The mitochondrial AAA metalloproteases are encoded by a small gene family that appears to include three or four members in metazoans as in fungi. In humans, three such genes apart from paraplegin have been partially characterized, namely AFG3L1 [17], AFG3L2 [18] and a gene most similar to yeast *YME1*, designated *YME1L* (Genbank AJ132637), which is located on chromosome 10p (see http://www.ncbi.nlm.nih.gov/cgi-bin/UniGene/clust?ORG=Hs&CID=206521). A similar gene has been reported in mouse (Genbank AF090430). The proposed human YME1L protein of 716 amino acid residues shows 42% identity to its yeast counterpart and contains the conserved ATPase and zinc-binding HEXXH domains. In this report we confirm, using a transiently expressed green fluorescent protein (GFP) reporter, that the human protein is mitochondrially targeted and we demonstrate that its expression in a *yme1* disruptant yeast strain restores growth on glycerol at 37°C. We propose that *YME1L* is a functional homologue of *YME1*, with conserved roles in mitochondrial assembly, integrity and perhaps DNA metabolism.

2. Materials and methods

2.1. Molecular cloning and reverse transcription-polymerase chain reaction (RT-PCR)

Standard techniques were used [19] with slight modifications where indicated. Restriction enzymes and DNA modifying enzymes were used under conditions recommended by the manufacturer. The *YME1L* coding sequence was amplified from HeLa cells first-strand cDNA using 5′ primer YME52 CTAGTCTAGAACTAGGGAGATGTTTTCCTTGTCGAGC (*YME1L* start codon underlined, *Xba*I cloning site double-underlined) and as 3′ primer one of YME32 CGGGTACCCGTCTCACTTCCAACTTTTTCCCCTCAA (comple-

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ment of last sense codon of *YME1L* underlined, *Kpn*I cloning site double-underlined), GJH02 GGGGCTGCAGCTATCTCACTTCCAACTTTTTCCCC (complement of last sense codon of *YME1L* plus synthetic stop codon underlined, *Pst*I cloning site double-underlined), and GJH01 CCCGGCGGCCGCCGGCGCGCCTCTCACTTCCAACTTTTTCCCC (complement of last sense codon of *YME1L* underlined, *Not*I cloning site double-underlined). PCR products were digested with *Xba*I plus the appropriate 3′ terminal enzyme prior to ligation, respectively, into pEGFP-N1, p425CYC1 [20] and a GFP-containing version of p425CYC1. The yeast *YME1* gene was amplified by PCR from genomic DNA of strain W303/1A [21] using primers MWG176 GGGACTAGTTTAATTAAGATGAACGTTTCAAAAATACTTG (*YME1* start codon underlined, *Pac*I cloning site double-underlined) and MWG177 CCCGGCGGCCGCCGGCGCGCCTGCATTTAACATTGTAGG (complement of the last sense codon of *YME1* underlined, *Not*I cloning site double-underlined). The PCR product was digested with *Pac*I and *Not*I prior to ligation into a modified version of p425CYC1 that introduces a C-terminal HA tag. Sequence analysis of the clones was performed by dye-terminator chemistry on the Perkin Elmer ABI 310 Genetic Analyzer, with kit reagents supplied by the manufacturer.

2.2. Expression in yeast

Yeast strains *Saccharomyces cerevisiae* W303/1A (*MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*) and W303ΔYME1 (*MATa ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 yme1-Δ1::URA3*) [13] were transformed by the one-step method [22]. For testing complementation yeast were replicated on YPGly (1% yeast extract, 1% peptone, 4% glycerol in 2.2% agar) plates and incubated at either 30 or 37°C. For GFP detection yeast colonies were inoculated in 1 ml YPGly and grown overnight at 37°C. The culture was then diluted to an OD_{600} of approximately 0.3–0.5 and DAPI was added to a final concentration of 0.2 μg/ml. The yeast transformants were grown for about one generation prior to analysis by means of fluorescence microscopy.

2.3. Expression in HeLa cells

HeLa cells were grown on glass coverslips and transiently transfected using Lipofectamine Reagent (Gibco BRL) according to the manufacturer's recommendations. After 24 h, cells were washed with Dulbecco's modified Eagle's medium and incubated in medium containing 100 nM Mitotracker Red (Molecular Probes) for 10 min. After two phosphate-buffered saline (PBS) washes, normal medium was replaced and the cells incubated for 2 h at 37°C. They were rewashed twice in PBS, fixed in 4% formaldehyde/5% sucrose in PBS for 10 min at 37°C, washed twice more in PBS then mounted on slides using Vectashield mounting medium (Vector Laboratories). The cells

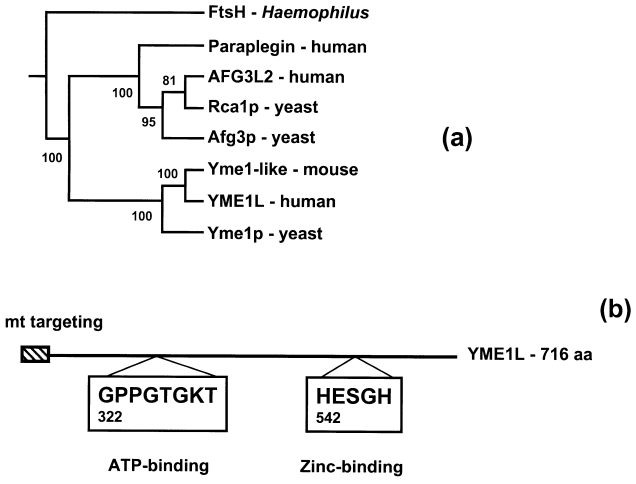

Fig. 1. Phylogenetic conservation of YME1L. (a) Protein parsimony tree created by PROTPARS, with bootstrap values for each node. Sequences used are FtsH (*Haemophilus influenzae*; Genbank AAC22979) as outgroup, plus yeast and proposed mammalian members of the mitochondrial AAA family as detailed in the main text. (b) Schematic diagram of major features of the YME1L protein (numbered from the N-terminal methionine), showing the 46 amino acid mitochondrial targeting signal predicted by MITOPROT (http://websvr.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) and PSORT (http://psort.nibb.ac.jp/form.html), plus the putative ATP- and zinc-binding motifs associated with ATPase and metalloprotease activities.

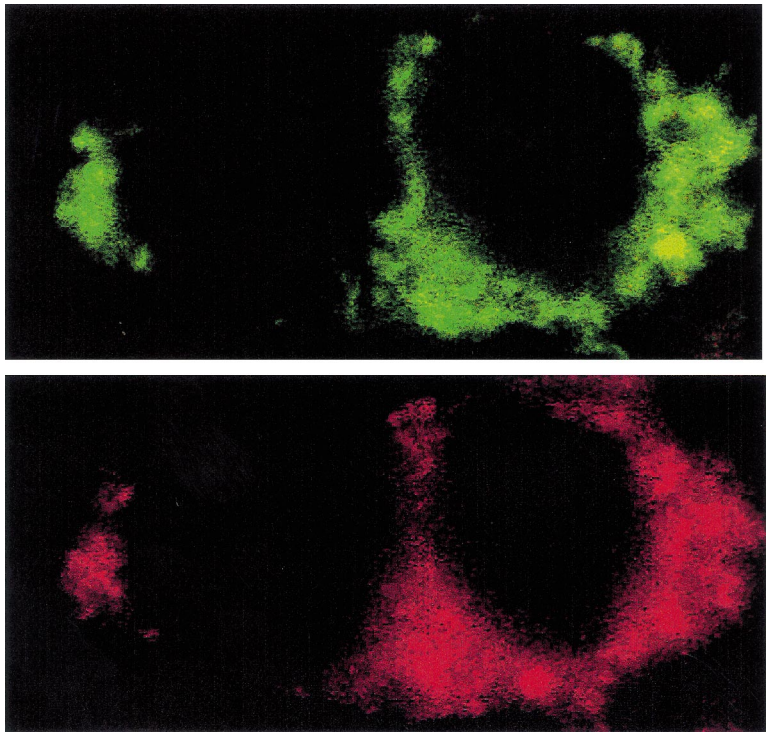

Fig. 2. Subcellular localization of YME1L-EGFP in HeLa cells. Fluorescence micrographs of cells transiently transfected with the YME1L-EGFP construct and counterstained with Mitotracker Red. EGFP expression is visible in filamentous cytoplasmic structures of two cells in the field (upper panel). Mitotracker Red stains the same structures in these cells, revealing them as mitochondria (lower panel).

were visualized under an Olympus S BX50 microscope, with appropriate filters for Mitotracker Red (U-MWG, wavelength 510–550 nm) and EGFP (U-MWB, wavelength 450–480 nm) fluorescence.

2.4. Phylogenetic analysis

Protein parsimony (PROTPARS) analysis used the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html).

3. Results and discussion

3.1. Molecular phylogeny of YME1L

Protein parsimony analysis of aligned polypeptide sequences gave the robust tree (all nodes relating to the position of YME1L 100% supported by bootstrap analysis) shown in Fig. 1. As expected, the closest relative of the YME1L polypeptide sequence from the set analyzed is the proposed mouse homologue (Genbank AF090430). Both of these are robustly placed together with yeast Yme1p, and separately from Afg3p, Rca1p and the other human homologues, AFG3L2 and paraplegin. Discounting likely sequencing errors and partially spliced transcripts, no other human cDNAs deposited in dBEST encode a protein highly similar to Yme1p. The *Caenorhabditis elegans* and *Drosophila* genomes also contain only a single close homologue of *YME1* (M.K. Juhola and H.T. Jacobs, unpublished data). We therefore propose that *YME1L* should be regarded as the human orthologue of yeast *YME1*.

3.2. Expression of YME1L in human cells

The human YME1L cDNA was cloned into the GFP reporter vector pEGFP-N1. Following transient transfection into HeLa cells, the YME1L-EGFP fusion protein was expressed clearly in only a minority of cells, compared with similar constructs for other mitochondrially targeted proteins, such as DNA polymerase γ (J.N. Spelbrink and H.T. Jacobs, unpublished data), or with an EGFP construct lacking mitochondrial targeting information completely. In cells where YME1L-EGFP fluorescence was detectable it was weak (the photographic image in Fig. 2 is enhanced to show the spatial distribution of signal). Most of the signal co-localized with mitochondria, based on co-staining with Mitotracker Red, but staining was more diffuse than seen typically, and many highly expressing cells were morphologically abnormal.

3.3. Expression of YME1L in yeast

To confirm mitochondrial localization of the YME1L protein in yeast, the human cDNA was ligated into a high copy-number yeast reporter vector, as an in-frame fusion to GFP, and introduced into yeast cells. GFP was seen in a typical mitochondrial pattern, although strong fluorescence (Fig. 3a) was detected in only a small minority of cells, and in these highest expressors DAPI fluorescence was weak (data not shown), suggesting that over-expression of the human protein in yeast may affect mitochondrial integrity or mtDNA levels. Untransformed yeast showed no green fluorescence, and cells

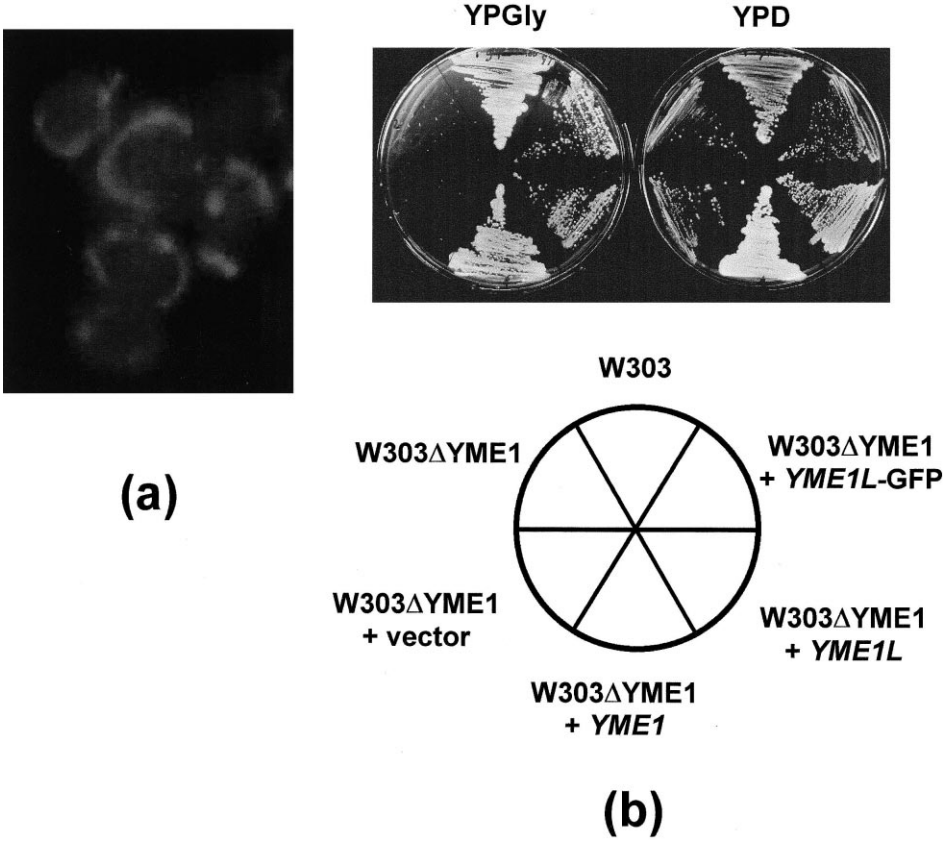

Fig. 3. Expression of YME1L protein in yeast. (a) Subcellular localization. Fluorescence microscopy of yeast cells, transformed with pGH16-YME1L (encoding YME1L-GFP). (b) Complementation of a *yme1* disruptant yeast strain. Yeast strain W303ΔYME1 was transformed with plasmids containing either yeast *YME1*, human *YME1L* (with and without fusion to GFP) and empty vector. Growth was on glycerol at 30 or 37°C as shown.

transformed with a GFP vector showed uniform, diffuse staining in the cytosol, with no evidence of specific localization to mitochondria (data not shown).

To study whether the human protein was able to complement the disruption of yeast *YME1*, transformants were grown under selective conditions lethal to *yme1* mutants, i.e. on glycerol at 37°C. The *yme1* strain grew on either glucose or glycerol at 30°C, whether or not it was transformed with human *YME1L*. The untransformed or vector-transformed strains failed to grow under selective conditions, but cells transformed with *YME1L*, either untagged or as a GFP fusion-protein, (Fig. 3b) grew under all conditions, albeit slower than the wild-type strain or the disruptant strain transformed by the yeast gene.

Previous reports showed that complementation of the *yme1* disruptant requires the Walker ATPase and zinc-binding HEXXH domains of Yme1p [13], both of which are conserved in YME1L (Fig. 1b). Overall, our data strongly indicate that *YME1L* is the functional homologue of yeast *YME1*. We propose that it plays a phylogenetically conserved role in mitochondrial integrity, and should be regarded as a strong candidate for involvement in human mitochondrial disorders.

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