The C-terminal extension domain of *Saccharomyces cerevisiae* MrpL32, a homolog of ribosomal protein L32, functions *in trans* to support mitochondrial translation

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Mitochondrial ribosomal protein L32 (MrpL32) of *Saccharomyces cerevisiae* is homologous to the bacterial L32 ribosomal protein. MrpL32 carries an N-terminal mitochondrion-targeting sequence (MTS) and is about 60 amino acid residues longer at the C-terminus. Adding to its function as a leader sequence, the MTS of MrpL32 has been reported to regulate ribosome biogenesis through its processing by m-AAA protease. However, the function of the C-terminal extension (CE) remains totally unknown. Therefore, we constructed a series of C-terminally truncated *mrpl32* (*mrpl32ΔC*) genes and expressed them in a *Δmrpl32* mutant to examine their function. Interestingly, some MrpL32ΔC derivatives exhibited temperature-sensitive (ts) growth on medium with non-fermentable carbon sources. Furthermore, the CE domain of MrpL32, expressed separately from MrpL32ΔC, could rescue the ts phenotype of mutants by improving mitochondrial protein synthesis.

Key words: C-terminal extension, mitochondrial ribosome, MrpL32

Many protein components of mitochondrial ribosomes are homologous to eubacterial ribosomal proteins, but they tend to be larger than the corresponding bacterial proteins (Graack and Wittmann-Liebold, 1998; Gan et al., 2002; Smits et al., 2007). Only a small number of the mitochondrial ribosomal proteins (MRPs) have been investigated extensively and the function of extended regions of these MRPs revealed. MrpL36 and MrpS28 in *Saccharomyces cerevisiae* are two such examples. MrpL36 is a homolog of bacterial L31 ribosomal protein (r-protein) and harbors a mitochondrion-specific domain at the C-terminus. This C-terminal extension (CE) of 62 amino acid residues (aa) is dispensable for mitochondrial translation *per se*, but is required for the suppression of specific *cox2* mutants and important for the stability of MrpL36 (Williams et al., 2004; Prestele et al., 2009). MrpS28, a mitochondrial homolog of S15 r-protein, has a unique domain of 117 aa at its N-terminus. In contrast to the CE of MrpL36, this extended region is essential and the core region, which is homologous to S15, alone cannot function for translation in mitochondria. Interestingly, however, this extended domain, when produced separately from the core region, was able to complement the inactive S15 domain *in trans*

(Huff et al., 1993).

MrpL32 is unique; its long mitochondrion-targeting sequence (MTS) (71 aa) is a target of m-AAA protease, and the loss of MrpL32 processing is thoroughly responsible for the growth failure of an m-AAA protease mutant on medium with non-fermentable carbon sources (Nolden et al., 2005). It was also indicated that the folding of MrpL32 halts cleavage initiated from the N-terminus by m-AAA protease and avoids its total degradation, and that for proper folding, the MTS sequence as well as a conserved sequence motif located at the end of the L32 r-protein domain are important (Bonn et al., 2011). However, the functional importance of the CE of MrpL32 (MrpL32-CE) has never been investigated. To examine this, we first constructed plasmids that express a serial C-terminally-truncated MrpL32. Plasmid pSH-LEU (Gan et al., 2002) was used as the vector and truncation sites were chosen considering the domain boundary and predicted secondary structure of the CE (Buchan et al., 2013) (Fig. 1A). We transformed a haploid *Δmrpl32* mutant (α :*Δmrpl32*/pXP722-MRPL32) with these plasmids, and then eliminated pXP722-MRPL32 from the transformants and examined their growth on medium with non-fermentable carbon sources (Fig. 1B) to assess the function of C-terminally-truncated versions of MrpL32 in mitochondrial translation. The haploid *Δmrpl32* mutant (α :*Δmrpl32*/pXP722-MRPL32) was obtained by

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Fig. 1. The CE domain of MrpL32 is required for normal mitochondrial translation and complements truncated MrpL32 *in trans*. (A) Schematic domain organization of MrpL32. MTS: mitochondrion-targeting sequence; L32: L32 r-protein domain; CE: C-terminal extension. Predicted secondary (2nd) structures, α -helices (gray ellipses) and β -strands (white rectangles), are depicted. Solid lines underneath indicate regions cloned on plasmid pSH-*LEU* or pVT100U-mtGFP. (B) $\Delta mrpL32$ mutants carrying the indicated versions of MrpL32 were cultured overnight in yeast synthetic medium. Serial ten-fold dilutions were spotted on YPGE (2% glycerol/2% ethanol) and YPD (2% glucose) plates, which were incubated for 4 and 2 days, respectively, at the indicated temperature. (C) Mutant strains containing the indicated MrpL32 Δ C and transformed with pVT100U-mtGFP (GFP) or pVT100U-mtMrpL32-CE (CE) were analyzed for respiratory growth as described in (B). (D) Steady-state level of Cox2 in pVT100U-mtGFP (GFP) or pVT100U-mtMrpL32-CE (CE) transformants of $\Delta mrpL32$ mutants containing MrpL32-FL (FL) or MrpL32-152 (152). Strains were cultured in YPGG (2% glycerol/2% galactose) at the indicated temperature, and lysates were prepared and analyzed by Western blotting using antibodies against Cox2 (Anti-MTCo2 [4B12A5], Abcam) and Arp3 (loading control) (Arp3(A-10), Santa Cruz Biotechnology).

sporulation of hetero-diploid strain Y23483 (*MRPL32/mrpL32::kanMX4*) carrying pXP722-*MRPL32*. Y23483 is a derivative of BY4743 (MATa/ α : *his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 LYS2/lys2 Δ 0 met15 Δ 0/MET15 ura3 Δ 0/ura3 Δ 0). Plasmid pXP722-*MRPL32* supplies full-length MrpL32 (MrpL32-FL) to support mitochondrial translation in the $\Delta mrpL32$ mutant and hence to maintain the genome in mitochondria, because it is known that cells defective in mitochondrial translation progressively lose their mitochondrial genomes and become permanently respiration-deficient. The Ura⁺ selection marker of vector pXP722 (Shen et al., 2012) enables plasmid elimination by simple selection of the survivors on medium containing 5-fluoroorotic acid (Boeke et al., 1987). The*

results obtained (Fig. 1B) showed that cells (α : $\Delta mrpL32$ /pSH-MrpL32-157) expressing MrpL32 Δ C of 157 aa (MrpL32-157) grew similarly well to those containing the wild-type protein (MrpL32-FL) on medium containing glycerol and ethanol, non-fermentable carbon sources (YPGE), which indicated that mitochondrial translation is active with MrpL32-157 and, hence, that the cells are respiration-competent. On the other hand, MrpL32-152, MrpL32-131 and MrpL32-120 supported respiratory growth of the $\Delta mrpL32$ mutant at 30 °C but not at 37 °C, and the mutant carrying plasmid pSH-MrpL32-120 exhibited very poor growth on YPGE even at 30 °C. MrpL32-120 lacks the C-terminal 2 aa of the predicted L32 core domain. The mutant carrying pSH-MrpL32-110 could

not grow at all on YPGE even at 30 °C, but grew well on medium containing glucose (YPD). Glucose is the primary and fermentable carbon source and allows budding yeast to grow without respiration; hence, mutant cells defective in mitochondrial translation can grow on YPD.

Because MrpL32-152 and MrpL32-131 proteins were active in mitochondrial translation but not at higher temperature, we next investigated whether the MrpL32-CE has a separate function as in the case of MrpL36 (Prestele et al., 2009) and can complement these MrpL32 Δ Cs *in trans*. The region of *MRPL32* encoding MrpL32-CE (121–183 aa) was amplified by PCR and cloned into pVT100U-mtGFP by replacing the GFP sequence, thereby appending the MTS of Su9 in *Neurospora crassa* (Ungermann et al., 1994) to MrpL32-CE. The resultant plasmid (pVT100U-mtMrpL32-CE) or pVT100U-mtGFP was used to transform Δ mrpL32 mutants expressing each MrpL32 Δ C and the growth phenotypes were examined (Fig. 1C). Interestingly, Δ mrpL32/pSH-MrpL32-131 and Δ mrpL32/pSH-MrpL32-152 transformed with pVT100U-mtMrpL32-CE grew at 37 °C on YPGE medium, albeit more slowly than cells containing MrpL32-157. On the other hand, transformants with pVT100U-mtGFP showed no growth on YPGE at 37 °C. The respiratory growth of Δ mrpL32/pSH-MrpL32-120 could not be complemented by MrpL32-CE. These results indicated that MrpL32-CE can function *in trans* but the L32 r-protein domain is essential for translation in mitochondria. There remained a possibility, however, that the recovery of temperature-resistant respiratory growth was due to wild-type *MRPL32* generated by homologous recombination between two fragments on different plasmids. To exclude this possibility, we examined the correlation of plasmid loss and ts respiratory growth as well as the structure of plasmids recovered from transformants. The results clearly showed that cells that lost a plasmid regained the ts phenotype, and that plasmids recovered from temperature-resistant transformants retained the original size of inserts (data not shown).

Finally, to investigate the mitochondrial translation of Δ mrpL32 mutants expressing MrpL32 Δ C and either MrpL32-CE or GFP, we extracted total proteins and examined the steady-state level of cytochrome oxidase subunit 2 (Cox2), which is encoded on the mitochondrial genome and translated by mitoribosomes. As shown in Fig. 1D, the Δ mrpL32 mutant carrying pSH-MrpL32-152 and pVT100U-mtGFP produced little, if any, Cox2 when cultured at 37 °C. On the other hand, Cox2 was clearly detected in cells carrying pVT100U-mtMrpL32-CE both at 30 °C and at 37 °C. Similar results were obtained with a Δ mrpL32 mutant carrying pSH-MrpL32-131 and either pVT100U-mtMrpL32-CE or pVT100U-mtGFP (data not shown). These results are consistent with the ts growth phenotype on YPGE medium, and indicate that MrpL32-CE indeed functions in mitochondrial translation *in trans*

with MrpL32-152 and MrpL32-131 peptides. The mechanism of complementation by MrpL32-CE of C-terminally-truncated MrpL32 remains to be elucidated. Stabilization of the produced L32 r-protein domain peptide is a possible explanation. It is also conceivable that MrpL32-CE assists the folding of the MrpL32 precursor and allows the m-AAA protease to process the MTS without degrading the whole protein. However, we could detect MrpL32 Δ C derivatives with the size expected for the mature protein in Δ mrpL32 mutants carrying only the corresponding pSH-MrpL32 Δ C (data not shown). Therefore, MrpL32-CE is not indispensable for processing by m-AAA protease. Alternatively, MrpL32-CE may have a separate function from the L32 r-protein domain. Previous work indicated that MrpL32 associates tightly with the mitochondrial inner membrane and that the assembly of the mitoribosome is completed in close proximity to the inner membrane (Nolden et al., 2005). Several membrane proteins such as Oxa1 anchor mitoribosomes to the inner membrane, but other components seem to participate in the membrane binding of the mitoribosome (De Silva et al., 2015). Therefore, MrpL32-CE may be functioning as such a component. In this context it is noteworthy that the gene for bacterial r-protein L32 is separated from the main r-protein gene loci on the chromosome and co-transcribed with membrane protein genes (Podkovyrov and Larson, 1995), although no apparent sequence homologies are observed between these membrane proteins and MrpL32-CE.

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