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Systematic Analysis of the Twin Cx_9C Protein Family

Sebastian Longen[1], Melanie Bien[1], Karl Bihlmaier[1], Christine Kloeppel[1], Frank Kauff[2], Miriam Hammermeister[3], Benedikt Westermann[3], Johannes M. Herrmann[1]* and Jan Riemer[1]

[1]*Cell Biology, University of Kaiserslautern, Erwin-Schrödinger-Strasse 13, 67663 Kaiserslautern, Germany*

[2]*Molecular Phylogenetics, University of Kaiserslautern, Erwin-Schrödinger-Strasse 13, 67663 Kaiserslautern, Germany*

[3]*Institute for Cell Biology, University of Bayreuth, 95440 Bayreuth, Germany*

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The Mia40–Erv1 disulfide relay system is of high importance for mitochondrial biogenesis. Most so far identified substrates of this machinery contain either two cysteine-x_3-cysteine (twin Cx_3C) or two cysteine-x_9-cysteine (twin Cx_9C) motifs. While the first group is composed of well-characterized components of the mitochondrial import machinery, the molecular function of twin Cx_9C proteins still remains unclear. To systematically characterize this protein family, we performed a database search to identify the full complement of Cx_9C proteins in yeast. Thereby, we identified 14 potential family members, which, with one exception, are conserved among plants, fungi, and animals. Among these, three represent novel proteins, which we named Cmc2 to 4 (for Cx_9C *m*otif-*c*ontaining protein) and which we demonstrated to be dependent for import on the Mia40–Erv1 disulfide relay. By testing deletion mutants of all 14 proteins for function of the respiratory chain, we found a critical function of most of these proteins for the assembly or stability of respiratory chain complexes. Our data suggest that already early during the evolution of eukaryotic cells, a multitude of twin Cx_9C proteins developed, which exhibit largely nonredundant roles critical for the biogenesis of enzymes of the respiratory chain in mitochondria.

Keywords: Erv1; Mia40; mitochondria; respiration; twin Cx_9C proteins

Introduction

Proteins of the intermembrane space (IMS) of mitochondria play important functions in energy metabolism; in the transfer of proteins, ions, and metabolites between the inner and the outer membrane; and in metal ion homeostasis (for review, see Refs. 1–4). All proteins of the IMS are encoded by nuclear genes. A number of proteins are targeted to the IMS by N-terminal presequences on a transport route that employs the translocases of the mitochondrial outer and inner membranes.[3,5,6] However, many proteins of the IMS, particularly those of low molecular mass, do not contain presequences but characteristic patterns of cysteine residues that mediate their import into mitochondria. Two groups of cysteine patterns have been identified:

1. Twin Cx_3C motifs: In these signatures, four cysteine residues form pairs each spaced by three amino acid residues. The terminal and central cysteine residues can form intramolecular disulfide bridges that stabilize a hairpin-like structure.[7,8] Twin Cx_3C motifs are characteristic for the small Tim proteins that facilitate the transport of hydrophobic membrane proteins from the outer to the inner membrane.[2,3,9] Fungi and animals consistently contain five structurally and functionally conserved small Tim proteins, namely, Tim8, Tim9, Tim10, Tim12, and Tim13.[10]
2. Twin Cx_9C motifs: In the second motif, the cysteine residues in the cysteine pairs are each spaced by nine residues. The best characterized protein of this group is Cox17, a copper-binding protein that plays a role in copper transfer to cytochrome *c* oxidase.[11,12] In Cox17, the twin Cx_9C motif stabilizes two antiparallel α-helices. Depending on the redox state of Cox17, these

**Corresponding author.* E-mail address: hannes.herrmann@biologie.uni-kl.de.

Abbreviations used: Cmc, Cx_9C motif-containing protein; IMS, intermembrane space; ORF, open reading frame; MISS, mitochondrial intermembrane space sorting.

opposing cysteine residues can be linked by disulfide bridges.[13] It was proposed that the Cox17-mediated copper transfer is associated with a change of the redox state of the protein.[14]

Besides Cox17, a number of additional proteins with twin Cx_9C motifs were identified and shown to reside in the IMS of mitochondria: Cmc1,[15] Cox19,[16] Cox23,[17] Som1,[18,19] Mdm35,[20,21] Mic14,[21] and Mic17.[21] Cmc1, Cox19, and Cox23 are required for biogenesis of cytochrome *c* oxidase. However, they lack a copper-binding site similar to the one found in Cox17 and their molecular function remains unclear. Furthermore, Som1-deficient mutants exhibit an impaired activity of the inner-membrane protease Imp1/Imp2, and the deletion of Mdm35 leads to an altered mitochondrial morphology.

In order to systematically characterize this conserved protein family, we identified all twin Cx_9C proteins encoded by the genome of *Saccharomyces cerevisiae*. This analysis revealed a complement of 14 proteins, most of which are conserved from plants to humans. Of the 14 proteins, three were novel twin Cx_9C family members, which we named Cmc2 to Cmc4. Whereas the deletions of the *CMC2* and *CMC3* genes are associated with defects in cytochrome *c* oxidase and respiratory chain activity, no defects were observed in mutants lacking *CMC4*. Our analysis suggests that twin Cx_9C proteins form a complex system of components crucial for the biogenesis of the enzyme complexes of the respiratory chain.

Results

S. cerevisiae contains 14 twin Cx_9C proteins

To identify the full complement of twin Cx_9C proteins of yeast, we searched for open reading frames (ORFs) potentially encoding proteins with twin Cx_9C signatures with no more than 100 residues between these motifs using the PatMatch tool of the *Saccharomyces* Genome Database.[22] This screen used similar parameters as that performed by Gabriel *et al.*[21] Eighty-six candidates were identified, which mainly fall into three groups strongly differing in their size distribution (Fig. 1). Most proteins exhibited molecular masses of more than 30,000. This group contained mainly transcription factors in which the cysteine residues were part of DNA-binding zinc fingers. A notable exception was Mia40, a member of the mitochondrial disulfide relay system.[23] A second group of proteins is constituted by four proteins of very small molecular mass (less than 8 kDa) that show a high content of cysteine residues (19–28%). Three of these proteins, Cup1-1, Cup1-2, and Crs5, were previously functionally characterized. They represent metallothioneins, which are involved in the metal homeostasis of the cell.[24,25] The fourth putative gene product, Yil046w-a, resembles the metallothioneins in size and cysteine content. However, homologs of this ORF are absent even in yeast species closely related to

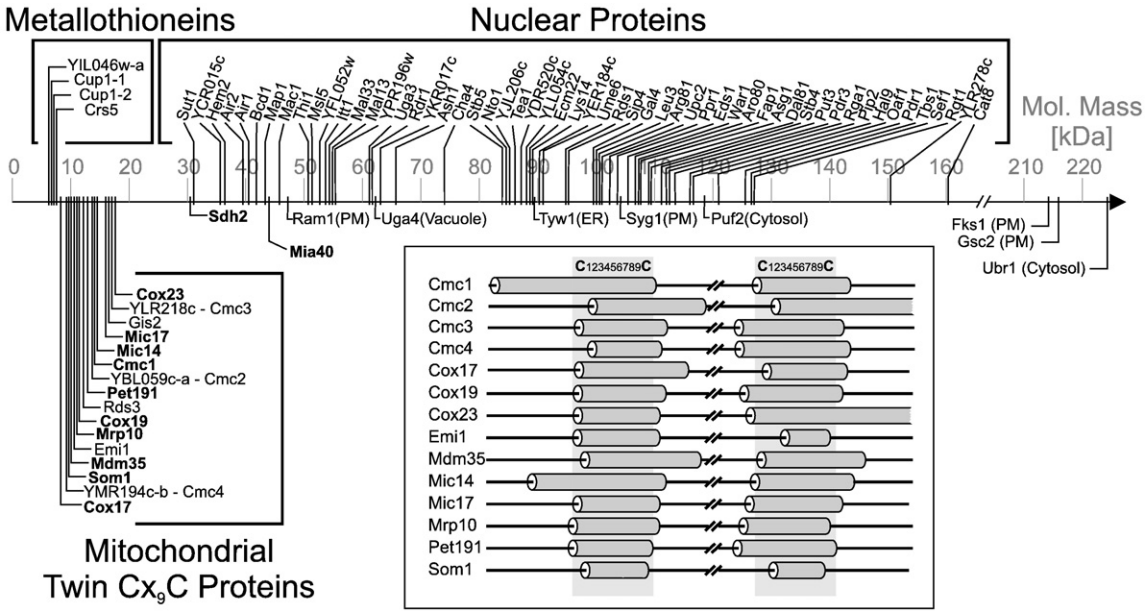

Fig. 1. The yeast genome codes for 86 proteins with twin Cx_9C motifs. Employing the PatMatch algorithm,[22] we searched the yeast genome for gene products containing two cysteine-x_9-cysteine patterns that are spaced by not more than 100 residues. In total, 86 proteins were identified, which are ordered on the basis of their molecular mass. Most of the proteins that are larger than 30 kDa are characterized transcription factors and/or contain nuclear localization signals. Four small proteins (less than 8 kDa) show a very high content of cysteine residues (19–28%), which is typical for metallothioneins. A third group of proteins in a size range of 9 to 18 kDa contains all so far identified mitochondrial twin Cx_9C proteins (shown in boldface) plus six additional ones (Cmc2 to Cmc4, Emi1, Gis2, and Rds3). Gis2 and Rds3 resemble typical zinc-finger proteins in their structure. Algorithms (see Materials and Methods for details) consistently predict helix–turn–helix structures for the 14 twin Cx_9C proteins proteins, as depicted in the box.