The twin-Cx9C motif protein Pet191 is essential for cytochrome c oxidase maturation. The motif Cys residues are functionally important and appear to be present in disulfide linkages within a large oligomeric complex associated with the mitochondrial inner membrane. The import of Pet191 differs from that of other twin-Cx9C motif class of proteins in being independent of the Mia40 pathway.

Cytochrome c oxidase (CcO), the terminal enzyme of the respiratory chain in mitochondria, consists of 12 or 13 subunits, with the 3 core enzyme subunits (Cox1 to Cox3) being encoded by the mitochondrial genome (3). The assembly of CcO requires a myriad of steps, including the insertion of heme α and copper cofactors. Copper insertion into newly synthesized Cox1 and Cox2 chains occurs on the intermembrane space (IMS) side of the inner membrane (1M), as the accessory molecules are localized within this compartment (10). Two proteins, Cox11 and Sco1, are associated with the IMS and mediate the copper metallation of the CuB and CuA sites in Cox1 and Cox2, respectively (7, 18). Cu(I) ions transiently bound by Cox11 and Sco1 are provided by Cox17 within the IMS (12). Cox17 contains a twin-Cx9C structural motif that adopts a helical hairpin conformation, stabilized by two disulfide bonds with a single Cu(I) ion bound by vicinal Cys residues outside the twin-Cx9C motif (1, 2, 5). Two other IMS proteins, Cox19 and Cox23, are structurally related to Cox17 in containing a twin-Cx9C structural motif and function in an undefined step in CcO assembly (19, 21).

The uncharacterized Pet191 protein is a variant of the twin-Cx9C motif family. The conservation of the twin-Cx9C motif in Pet191 (see Fig. S1 in the supplemental material) and its importance in respiration motivated us to investigate the role of Pet191 in CcO assembly (15). Saccharomyces cerevisiae strains lacking Pet191 are known to be deficient on respiration and fail to propagate on growth medium containing glycerol as the sole carbon source (15) (Fig. 1A). The mutant cells are rho−, as a 3′ Myc-tagged PET191 gene can restore respiratory function. CcO activity was absent from cells cultured on glucose or raffinose, but succinate can restore respiratory function. CcO activity was absent in certain CcO assembly mutants (14). The specific defect in CcO was demonstrated by blue native polyacrylamide gel electrophoresis (BN-PAGE), as dimeric complex III and both monomeric and dimeric complex V species were observed in pet191Δ cells (Fig. 1C). Complex IV was absent, as visualized by Cox2 immunoblotting. Steady-state levels of Cox1, Cox2, and Cox3 were undetectable in pet191Δ cells (Fig. 1D). The diminution in Cox1 to Cox3 protein levels in pet191Δ cells arises from the impaired stability of CcO, since mitochondrial translation is comparable to what is seen for other CcO assembly mutants (4).

Whereas the twin-Cx9C protein Cox17 has a role in copper metallation of CcO during biogenesis, Pet191 does not appear to have a prominent role in this process. The addition of supplemented copper salts to the growth medium of pet191Δ cells does not reverse the respiratory function-deficient phenotype, as occurs with cox17Δ cells. Copper ions used in the metallation of CcO and Sod1 in the IMS derive from the matrix copper-ligand complex (9). Cells lacking Pet191 have normal mitochondrial copper levels and normal Sod1 activity in mitochondria, suggesting that Pet191 does not perturb mitochondrial copper metallation processes or Sod1 activation within the IMS. Immunoblotting of Myc-tagged Pet191 revealed that it localizes to the mitochondria (Fig. 1E) and was tightly associated with a membrane (Fig. 1F). Pet191 was not solubilized by sonication of the mitoplasts and was not released from the IM by sodium carbonate extraction at pH 10.5. However, at pH 11.5, sodium carbonate buffer was sufficient to solubilize Pet191. Pet191 remained associated with mitoplasts after hypotonic swelling but was degraded with the addition of protease K (Fig. 1G). The release of the IMS Cyb2, but not Pet191, upon hypotonic swelling suggested that Pet191 is not a soluble IMS protein. Thus, Pet191 is tightly associated with the IM facing the IMS side of the membrane.

Chromosomally HA-tagged Pet191 solubilized in digitonin migrated on BN-PAGE gels as a complex of approximately 500 kDa (Fig. 2A). Deoxycholate (DOC)-solubilized
Pet191-Myc eluted upon size permeation chromatography at a volume corresponding to approximately 530 kDa (Fig. 2C). However, extraction of Pet191 with 0.1% DOC in the presence of 100 mM dithiothreitol (DTT) resulted in elution of Pet191 at a volume closer to the predicted monomeric mass (Fig. 2C). These results are consistent with Pet191 existing in an oxidized conformer in mitochondria. CcO-deficient cells contain a more reducing IMS, as assessed by the Mia40 redox state (6). To determine whether the Pet191 oligomer was sensitive to perturbations in the redox state of the IMS, BN-PAGE analysis was carried out on Pet191-HA in respiratory function-deficient \( \text{cox}11 \) /H9004 cells cultured in raffinose. The Pet191 oligomer persisted, albeit at lower levels in \( \text{cox}11 \Delta \) cells (Fig. 2B).

Mutational analysis of Pet191 was carried out to assess whether the cysteine residues are functionally important. Cysteinyl residues within the twin-Cx9C motif as well as the linker motif were singly substituted with alanine residues (Fig. 3B). Mutant alleles of \( \text{PET}191 \) were transformed into \( \text{pet}191 \) /H9004 cells and tested for their ability to support growth on glycerol-containing medium. Cells harboring C5A and C56A mutant alleles were respiratory function deficient, whereas three additional alleles, the C15A, C32A, and C46A alleles, were partially compromised in growth at 30°C or 37°C (Fig. 3A).
Pet191 folds in a helical hairpin in a manner analogous to that seen for Cox17 or Cox12, then Cys5 and Cys56 may be an aligned pair existing as a disulfide bridge (Fig. 3B). All mutant proteins were equivalently expressed, as shown by immunoblot analysis (Fig. 3C).

Transformation of WT cells with the mutant PET191 alleles revealed that the presence of either C5A or C56A Pet191 had a slight dominant negative effect on respiratory growth (Fig. 3D). In contrast, the C15A mutant, which was only weakly compromised in supporting glycerol growth of pet191Δ cells, lacked any negative effects on the growth of WT cells on glycerol medium. The C5A mutant protein existed in a complex that was small relative to that for the WT protein, as determined by gel filtration (Fig. 3E). Thus, the nonfunctionality of the C5A protein may result from an abnormal Pet191 complex. To determine whether the dominant negative effect of C5A Pet191 influenced the endogenous Pet191, we carried out gel filtration studies on WT cells harboring the Csa Pet191 mutant. The presence of Csa Pet191 in WT cells led to an attenuation in the size of the solubilized WT protein (Fig. 3E).

The dominant negative effect of the mutant Pet191 on the WT protein suggested that the two proteins interact. This was confirmed by immunoprecipitation (IP) studies. Mitochondria isolated from cells harboring a vector encoded Pet191-Myc and chromosomal Pet191-HA were used for IP with anti-Myc beads. Pet191-HA exhibited co-IP with Pet191-Myc (Fig. 3F). Thus, Pet191 complex is a homo-oligomer, but the large size of the complex, ~500 kDa, may suggest that additional proteins are present.

Two of the six conserved Cys residues (Cys5 and Cys56) important for Pet191 function may participate in the disulfide stabilization of the complex, since the Csa Pet191 allele product fails to assemble into the WT complex. Structures of three twin-Cx9C motif proteins, Cox12, Cox17, and Qcr6, reveal disulfide-bonded helical hairpin conformations. Cox12 and Qcr6 are IMS-facing subunits of the CO and bc1 complexes, respectively. In S. cerevisiae, only one of the two Cys pairs exists in Qcr6. If Pet191 adopts a related helical hairpin conformation, the functionally important Cys residues Cys5 and Cys56 may form a disulfide pair.

Twin-Cx9C motif proteins like Cox17 are imported into the IMS by the MIA import pathway through an oxidative folding mechanism involving Mia40 and Erv1 (8, 16, 22, 24). Since Pet191 has a related twin-Cx9C motif, we addressed if Pet191 was imported through the MIA pathway. Temperature-sensitive erv1-2ts mutant (13) cells cultured at 22°C import IMS proteins normally, but import is attenuated upon a shift of cells to the nonpermissive temperature (16, 22). We observed that erv1-2ts cells cultured at 22°C have normal levels of Sod1, Ccs1, Cox23, and Pet191 within the mitochondria (Fig. 4A). However, cells shifted to 37°C have attenuated levels of Sod1, Ccs1, and Cox23 but not Pet191-Myc, suggesting that Pet191 is imported in a MIA-independent pathway. The attenuated levels of Sod1, Ccs1, and Cox23 are consistent with their dependency on the MIA complex for IMS import. An independent assessment of the role of Erv1 in Pet191 uptake was conducted using in vitro mitochondrial import of Pet191 translated in a rabbit reticulocyte lysate. Mitochondria were isolated from WT or erv1-2ts cells and tested for 35S-labeled Pet191 import. After treatment with proteinase K, Pet191 was observed in both WT and erv1-2ts mitochondria incubated at the nonpermissive temperature, at which Erv1 is inactive (Fig. 4B). Whereas the import of Pet191 into erv1-2ts mitochondria was normal, the import of radiolabeled Cox19, a known MIA substrate, was impaired in the respective mutant. The import of Cox19 was also normal in mitochondria isolated...
from pet191Δ cells (Fig. 4C). Thus, we conclude that Pet191 is imported into the mitochondria independent of Mia40/Erv1. The actual mechanism of Pet191 import is unclear, as it lacks an N-terminal mitochondrial import motif, as deduced by either the MITOPROT or the PSORT algorithm.

Pet191 joins the list of twin-Cx9C motif proteins that are...
involved in COX biogenesis, i.e., Cox17, Cox19, and Cox23. A series of other twin-Cx6C proteins whose functions are unknown exist within the IMS. These proteins include Mic14 and Mic17 (11). Although Mic14 contains a duplicated twin-Cx6C structural motif, it has no role in COX biogenesis. Cells lacking Mic14 show no growth defect on glycerol/lactate medium, consume oxygen at WT levels, and have normal COX activity. Thus, only a subset of soluble twin-Cx6C proteins in the IMS have roles in COX biogenesis.

The conservation of Pet191 in mammalian cells suggests that Pet191 may have a significant role in mammalian mitochondria. Functional studies on the human Pet191 ortholog have not appeared, nor have human mutations in PET191 been identified for patients with COX deficiency (23).

This work was supported by grant ES 03817 from the National Institutes of Environmental Health Sciences, NIH, to D.R.W.

We acknowledge the support of the CEMH core facility for fast-protein liquid chromatography (DK P30 072437). We acknowledge the assistance of Nataliya Zahayko. This paper is dedicated to the memory of Volodymyr P. Khalimonchuk.

REFERENCES

1. Abajian, C., L. A. Yatsunyuk, B. E. Ramirez, and A. C. Rosenzweig. 2004. Yeast Cox17 solution structure and copper(II) binding. J. Biol. Chem. 279:53354–53359.

2. Armesano, F., E. Balatri, L. Bacci, I. Bertini, and D. R. Winge. 2005. Folding studies of Cox17 reveal an important interplay of cysteine oxidase and copper binding. Structure 13:713–722.

3. Barrientos, A., M. H. Barros, I. Valnot, A. Rotig, P. Rustin, and A. Tzagoloff. 2002. Cytochrome oxidase in health and disease. Gene 286:53–63.

4. Barrientos, A., A. Zambrano, and A. Tzagoloff. 2004. MexS1p and Cox14p jointly regulate mitochondrial Cox1p expression in Saccharomyces cerevisiae. EMBO J. 23:3472–3482.

5. Barros, M. H., A. Johnson, and A. Tzagoloff. 2004. Cox23, a homologue of COX17, is required for cytochrome oxidase assembly. J. Biol. Chem. 279:31943–31947.

6. Bihlmayer, K., N. Mesecke, N. Terziyska, M. Bien, K. Hell, and J. M. Herrmann. 2007. The disulfide relay system of mitochondria is connected to the respiratory chain. J. Cell Biol. 176:759–772.

7. Carr, H. S., G. N. George, and D. R. Winge. 2002. Yeast Cox11, a protein essential for cytochrome c oxidase assembly, is a Cu(I) binding protein. J. Biol. Chem. 277:31237–31242.

8. Chacinska, A., S. Pfannschmidt, N. Wiedemann, Y. Kozjak, L. K. Sanjuan Szklarz, A. Schulze-Specking, K. N. Truscott, B. Guiard, C. Meisinger, and N. Pfanner. 2004. Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. EMBO J. 23:3735–3746.

9. Cobine, P. A., F. Pierrel, M. L. Bestwick, and D. R. Winge. 2006. Mitochondrial matrix copper complex used in metallation of cytochrome oxidase and superoxide dismutase. J. Biol. Chem. 281:36552–36559.

10. Cobine, P. A., F. Pierrel, and D. R. Winge. 2006. Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. Biochim. Biophys. Acta 1763:759–772.

11. Cojocarska, A., S. Pfannschmidt, N. Wiedemann, Y. Kozjak, L. K. Sanjuan Szklarz, A. Schulze-Specking, K. N. Truscott, B. Guiard, C. Meisinger, and N. Pfanner. 2004. Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. EMBO J. 23:3735–3746.

12. Cobine, P. A., F. Pierrel, M. L. Bestwick, and D. R. Winge. 2006. Mitochondrial matrix copper complex used in metallation of cytochrome oxidase and superoxide dismutase. J. Biol. Chem. 281:36552–36559.

13. Lange, H., T. Lisowsky, J. Gerber, U. Muhlenhoff, G. Kispat, and R. Lill, 2001. An essential function of the mitochondrial sulfredoxin oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. EMBO Rep. 2:715–720.

14. Maznchek, G., B. Repetto, D. M. Glerum, C. Jin, and A. Tzagoloff. 1997. SHY1, the yeast homolog of the mammalian SURF1 gene, encodes a mitochondrial protein required for respiration. J. Biol. Chem. 272:35342–35350.

15. Mumberg, D., R. Muller, and M. Funk. 1994. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use of heterologous expression. Nucleic Acids Res. 22:5767–5768.

16. Mittle, T., G. N. George, and D. R. Winge. 2004. Yeast Cox11, a protein essential for cytochrome c oxidase function is a Cu(I)-binding protein. J. Biol. Chem. 279:42520–42526.

17. Nobrega, M. P., S. C. B. Bandeira, J. Beers, and A. Tzagoloff. 2004. Studies of COX16, COX19 and COX23 genes required for cytochrome oxidase assembly in Saccharomyces cerevisiae. Curr. Genet. 43:8–19.

18. Mesecke, N., N. Terziyska, C. Kozany, F. Baumann, W. Neupert, K. Hell, and J. M. Herrmann. 2005. A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. Cell 121:1046–1056.

19. Mesecke, N., N. Terziyska, C. Kozany, F. Baumann, W. Neupert, K. Hell, and J. M. Herrmann. 2005. A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. Cell 121:1046–1056.

20. Mereu, C. M., R. Muller, and M. Funk. 1994. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use of heterologous expression. Nucleic Acids Res. 22:5767–5768.

21. Monné, D., R. Muller, and M. Funk. 1994. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use of heterologous expression. Nucleic Acids Res. 22:5767–5768.

22. Nöthig, E., C. S. B. Bandeira, J. Beers, and A. Tzagoloff. 2002. Characterization of COX19, a widely distributed gene required for expression of mitochondrial cytochrome c oxidase. J. Biol. Chem. 277:40206–40211.

23. Pierrel, F., M. L. Bestwick, P. A. Cobine, O. Khalimonchuk, J. A. Cricco, and D. R. Winge. 2007. Coa1 links the Mss51 post-translational function to Cox1 and Cox11, a protein essential for oxidase complex and assembly of cytochrome oxidase. J. Biol. Chem. 282:10233–10242.

24. Rissler, M., N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, and A. Chacinska. 2005. The essential mitochondrial protein Erv1p/cofactor insertion in cytochrome c oxidase assembly. EMBO J. 24:3435–3446.

25. Rigby, K., L. Zhang, P. A. Cobine, G. N. George, and D. R. Winge. 2007. Characterization of the cytochrome c oxidase assembly factor Cox19 of Saccharomyces cerevisiae. J. Biol. Chem. 282:10233–10242.

26. Rissler, M., N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, and A. Chacinska. 2005. The essential mitochondrial protein Erv1p cooperates with Mia40 in biogenesis of intermembrane proteins. J. Mol. Biol. 352:485–492.

27. Say, S. H. C., N. Cesti, M. Mancuso, E. A. Schon, S. Schanske, E. Bonilla, M. M. Davidson, and S. DiMauro. 2004. Studies of COX16, COX19 and PET191 in human cytochrome c oxidase deficiency. Arch. Neurol. 61:1935–1940.

28. Terziyska, N., T. Lutz, C. Kozany, D. Mokranjac, N. Mesecke, W. Neupert, J. M. Herrmann, and K. Hell. 2005. Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions. J. Biol. Chem. 280:579179–184.

29. Wittig, I., H. P. Braun, and H. Schagger. 2006. Blue native PAGE. Nat. Protoc. 1:418–428.