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NOTE

Pet191 Is a Cytochrome *c* Oxidase Assembly Factor in *Saccharomyces cerevisiae*^{∇†}

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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 *a* and copper cofactors. Copper insertion into newly synthesized Cox1 and Cox2 chains occurs on the intermembrane space (IMS) side of the inner membrane (IM), as the accessory molecules are localized within this compartment (10). Two proteins, Cox11 and Sco1, are associated with the IM and mediate the copper metallation of the Cu_B and Cu_A 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 cytochrome *c* reductase activity was elevated above wild-

type (WT) levels (Fig. 1B). Complex III activity is elevated 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 proteinase 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

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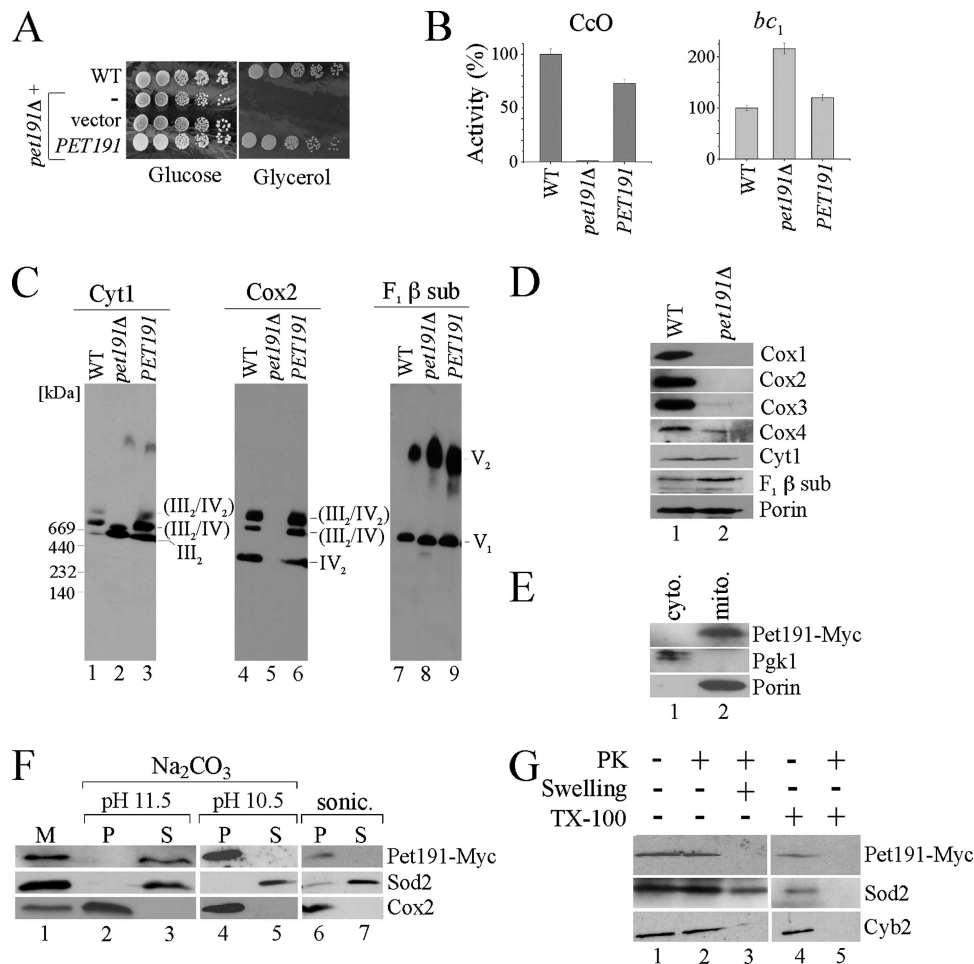


FIG. 1. Pet191 is required for CcO biogenesis. (A) Serial dilutions of *pet191Δ* cells transformed with an empty vector or *PET191* were plated onto complete medium containing 2% glucose or 2% lactate/glycerol as a carbon source. The WT and *pet191Δ* diploid strains of *Saccharomyces cerevisiae* (BY4743) were used. (B) CcO and SDH/*bc₁* activities. Activities of the WT mitochondria were set as 100%. Mitochondria were isolated from cells cultured on raffinose medium. (C) Mitochondria were solubilized in a lysis buffer containing 1.5% digitonin and protein complexes were separated by BN-PAGE. The respiratory chain complexes (III, IV, and V) were analyzed by immunoblotting with respective antibodies. sub, subunit. (D) Steady-state levels of mitochondrial proteins were analyzed by sodium dodecyl sulfate (SDS)-PAGE followed by Western blotting. (E) Cells expressing Pet191-Myc were fractionated, and cytosolic (cyto.) and mitochondrial (mito.) fractions were collected, resolved by SDS-PAGE, and analyzed by immunoblotting with the anti-Myc, anti-Pgk1, and anti-porin antibodies. The Pet191-Myc was expressed on a pRS426 vector under the *MET25* promoter and the *CYC1* terminator (17). (F) Mitochondria of a strain expressing Pet191-Myc (M; lane 1) were treated with either 0.1 M Na₂CO₃ (pH 10.5 or 11.5) or sonicated (sonic.) and fractionated by high-speed centrifugation. Obtained pellet (P; lanes 2, 4, and 6) and supernatant (S; lanes 3, 5, and 7) fractions were analyzed by immunoblotting using antibodies against the Myc epitope, matrix protein Sod2, and IM-anchored protein Cox2. (G) Intact (lanes 1, 2, and 4) or swollen (lane 3) mitochondria were incubated with (lanes 2, 3, and 5) or without (lanes 1 and 4) proteinase K (PK). In lanes 4 and 5, mitochondria were lysed with 1% Triton X-100 (TX-100) prior to incubation with PK. Western blot analysis was performed using antibodies specific for the Myc tag, matrix protein Sod2, and IMS protein Cyb2.

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 *cox11Δ* cells cultured in

raffinose. The Pet191 oligomer persisted, albeit at lower levels in *cox11Δ* cells (Fig. 2B).

Mutational analysis of Pet191 was carried out to assess whether the cysteine residues are functionally important. Cysteiny residues within the twin-Cx₂C motif as well as the linker motif were singly substituted with alanine residues (Fig. 3B). Mutant alleles of *PET191* were transformed into *pet191Δ* 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). If

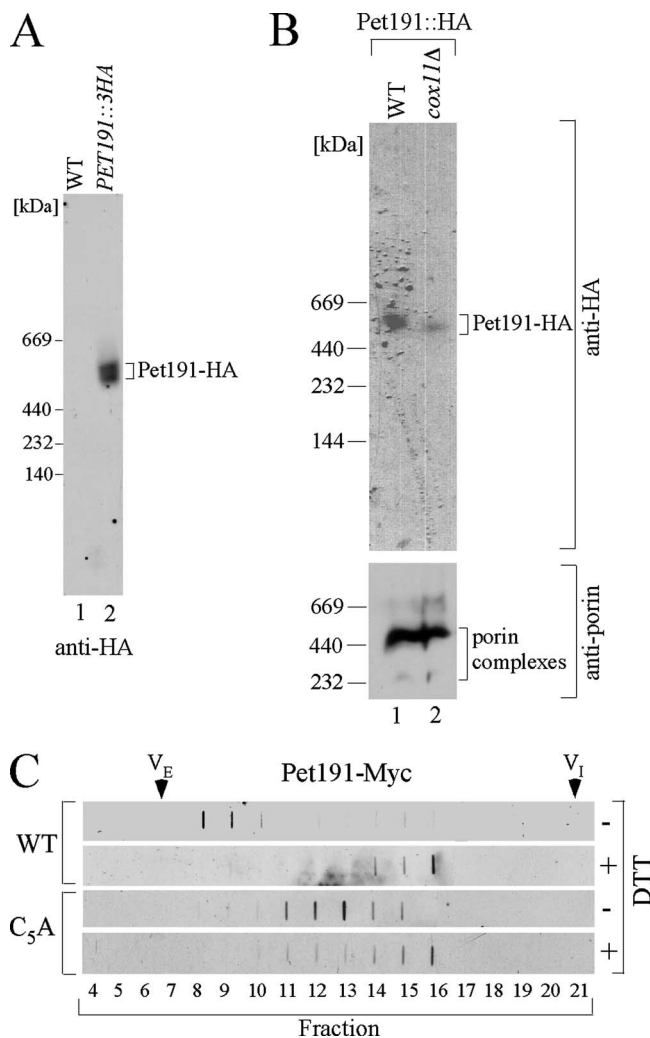


FIG. 2. Molecular size of Pet191. (A) Mitochondria isolated from WT cells and the strain containing genomically tagged Pet191 (carrying *PET191::3HA*) were solubilized and subjected to BN-PAGE followed by immunoblot analysis with an antibody against the HA epitope. A genomically HA-tagged variant of *PET191* was generated by homologous recombination, inserting the triple HA tag 3' to the open reading frame. (B) Mitochondria prepared from WT and *cox11Δ* cells carrying *PET191::3HA* were analyzed by BN-PAGE as described previously (25) using anti-HA and porin antibodies. (C) Cells expressing Pet191-Myc or its mutant form (C₅A) were used for mitochondrial preparation. Isolated mitochondria were lysed in a buffer containing 0.1% DOC in the presence (+) or absence (-) of 100 mM DTT, and clarified lysates were loaded onto the size exclusion column (10/30 G-200; Superdex) equilibrated with buffer containing 0.05% DOC \pm 5 mM DTT. Fractions were directly assayed by slot immunoblotting with anti-Myc antibodies. Column void (V_E) and internal (V_I) volumes are marked by arrows.

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 C5A Pet191 mutant. The presence of C5A 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, \sim 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 C5A Pet191 allele product fails to assemble into the WT complex. Structures of three twin-Cx₂C motif proteins, Cox12, Cox17, and Qcr6, reveal disulfide-bonded helical hairpin conformations. Cox12 and Qcr6 are IMS-facing subunits of the CcO and *bc*₁ 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-Cx₂C 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-Cx₂C 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

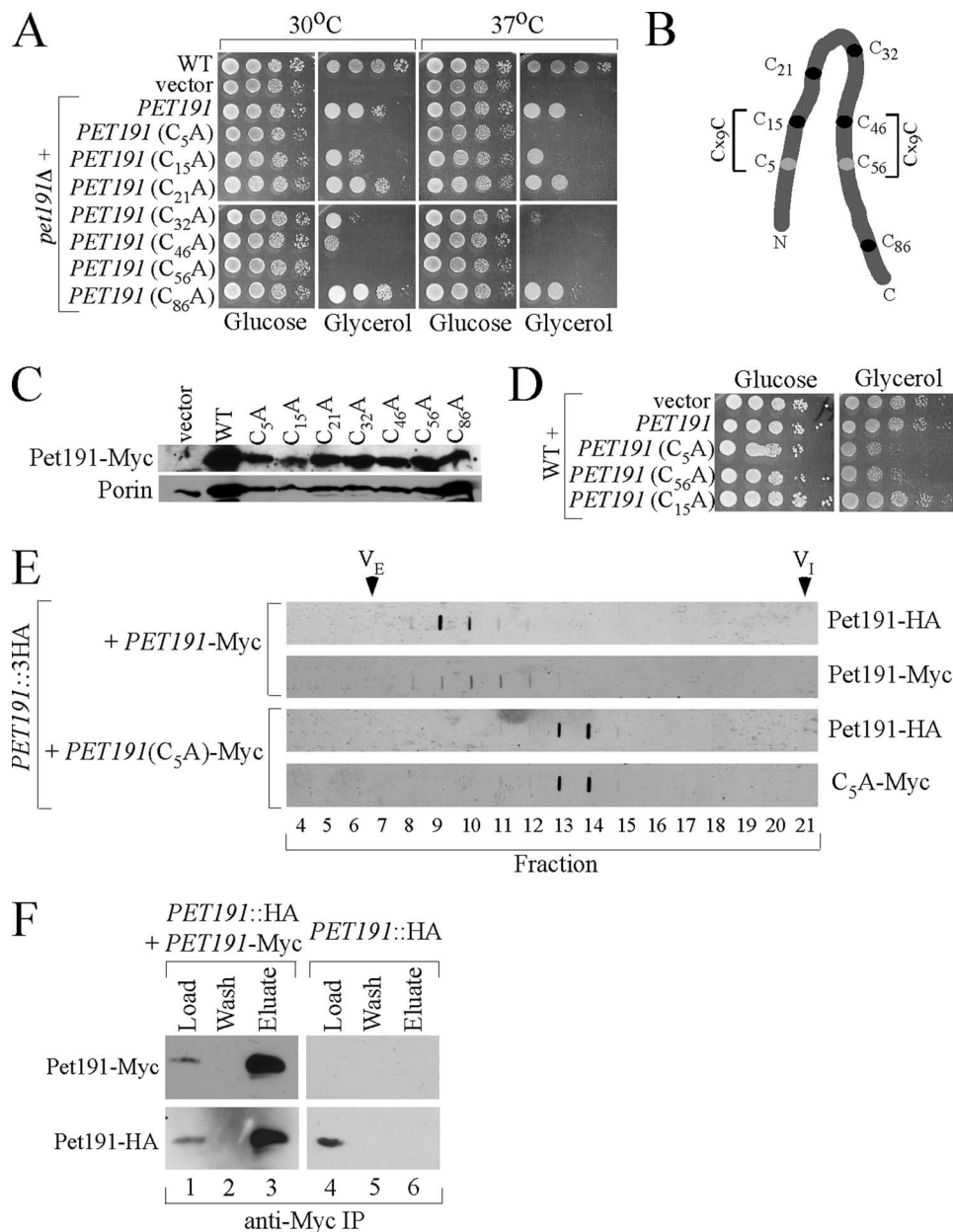


FIG. 3. Conserved cysteine residues are important for Pet191 function. (A) Serial dilutions of *pet191Δ* cells transformed with an empty vector, either *PET191* or one of its mutant forms, were plated onto complete medium containing 2% glucose or 2% lactate/glycerol as a carbon source and grown for 4 days at 30°C or 37°C. Mutant forms of Pet191 (the C₅A, C₁₅A, C₂₁A, C₃₂A, C₄₆A, C₅₆A, and C₈₆A mutants) were generated by site-directed mutagenesis using pRS426-*PET191*-Myc as a template. (B). Schematic demonstrating possible conformation of the Pet191 molecule where two Cx₉C motifs face each other. Positions of the most critical cysteine residues are depicted by light gray circles. Other Cys residues are shown in black. (C) Mitochondria isolated from *pet191Δ* cells carrying an empty vector, *PET191*, or its mutant forms were subjected to SDS-PAGE followed by Western blot analysis with the antibodies against the Myc epitope and porin. (D) WT cells overexpressing *PET191* or its C₅A, C₁₅A, or C₅₆A mutant versions were serially diluted, plated as described for panel A, and grown for 3 days at 30°C. (E) Mitochondria from the cells coexpressing Pet191-HA and Pet191-Myc or its C₅A mutant form were solubilized and analyzed as described above, except that DTT treatment was not included. Collected fractions were analyzed using anti-Myc and anti-HA antibodies. (F) Mitochondria isolated from the WT *PET191::3HA* strain with (lanes 1 to 3) or without (lanes 4 to 6) Pet191-Myc were lysed, and clarified extracts were immunoprecipitated with agarose-coupled anti-Myc antibodies. Co-IP of Pet191-Myc and Pet191-HA was performed as described previously (20), using anti-Myc-agarose-coupled beads (Santa Cruz Biotechnology). The load (representing 5% of the extracts; lanes 1 and 4), entire-wash (lanes 2 and 5), and eluate (lanes 3 and 6) fractions were analyzed by immunoblotting.

from *pet191Δ* cells (Fig. 4C). Thus, we conclude that Pet191 is imported into the mitochondria independent of Mia40/Ervi1. 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

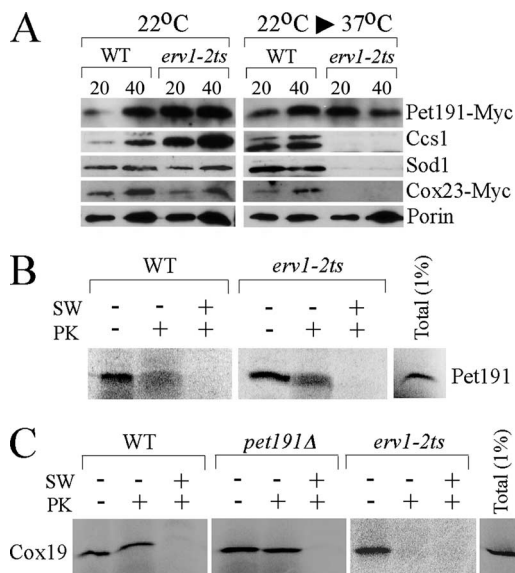


FIG. 4. *erv1* mutant mitochondria have normal levels of Pet191-Myc. (A) WT and temperature-sensitive *erv1-2ts* strains expressing Pet191-Myc or Cox23-Myc were grown at 22°C. The temperature-sensitive strain *erv1-2ts* (13) and the corresponding WT strain were a kind gift from Roland Lill. For isolation of mitochondria from *erv1-2ts* cells, the cells were pregrown in supplemented synthetic medium containing 2% raffinose at 22°C until the early logarithmic phase. Cultures were split into two halves, one of which was subjected to a temperature shift (22°C to 37°C), while the other was left at 22°C. After 7 to 10 h of incubation, cells were harvested and mitochondria isolated as described above. Mitochondria were isolated, separated by SDS-PAGE, and analyzed by immunoblotting with antibodies recognizing Myc-tag, Ccs1, Sod1, and porin. (B) Pet191 was synthesized in rabbit reticulocyte lysate in the presence of [³⁵S]methionine and incubated for 20 min at 30°C with mitochondria isolated from either WT or *erv1-2ts* cells. After incubation, mitochondria were reisolated, washed, and either swollen in hypotonic medium (SW) or incubated with proteinase K (PK). Samples were then subjected to SDS-PAGE followed by autoradiography. (C) Radiolabeled Cox19 was imported into the WT, *pet191Δ*, and *erv1-2ts* mitochondria as described above.

involved in CcO biogenesis, i.e., Cox17, Cox19, and Cox23. A series of other twin-Cx₉C proteins whose functions are unknown exist within the IMS. These proteins include Mic14 and Mic17 (11). Although Mic14 contains a duplicated twin-Cx₉C structural motif, it has no role in CcO biogenesis. Cells lacking Mic14 show no growth defect on glycerol/lactate medium, consume oxygen at WT levels, and have normal CcO activity. Thus, only a subset of soluble twin-Cx₉C proteins in the IMS have roles in CcO 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 CcO deficiency (23).

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