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Identification of amino acid residues of mammalian mitochondrial phosphate carrier important for its functional expression in yeast cells, as achieved by PCR-mediated random mutation and gap-repair cloning

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ABSTRACT

The mitochondrial phosphate carrier (PiC) of mammals, but not the yeast one, is synthesized with a presequence. The deletion of this presequence of the mammalian PiC was reported to facilitate the import of the carrier into yeast mitochondria, but the question as to whether or not mammalian PiC could be functionally expressed in yeast mitochondria was not addressed. In the present study, we first examined whether the defective growth on a glycerol plate of yeast cells lacking the yeast PiC gene could be reversed by the introduction of expression vectors of rat PiCs. The introduction of expression vectors encoding full-length rat PiC (rPiC) or rPiC lacking the presequence (ΔNrPiC) was ineffective in restoring growth on the glycerol plates. When we examined the expression levels of individual rPiCs in yeast mitochondria, ΔNrPiC was expressed at a level similar to that of yeast PiC, but that of rPiC was very low. These results indicated that ΔNrPiC expressed in yeast mitochondria is inert. Next, we sought to isolate "revertants" viable on the glycerol plate by expressing randomly mutated ΔNrPiC, and obtained two clones. These clones carried either of two mutations, F267S or F282S; and these mutations restored the transport function of ΔNrPiC in yeast mitochondria. These two Phe residues were conserved in human carrier (hPiC), and the transport function of ΔNhPiC expressed in yeast mitochondria was also markedly improved by their substitutions. Thus, substitution of F267S or F282S was concluded to be important for functional expression of mammalian PiCs in yeast mitochondria.

1. Introduction

Mitochondrial ATP synthesis is achieved in the matrix space owing to the electrochemical gradient of H^+ across the mitochondrial inner membrane acting as a driving force. To enable efficient ATP synthesis, the mitochondrial inner membrane shows high resistance against the permeation of molecules. However, numbers of molecular species involved in biochemical reactions such as ATP synthesis, β-oxidation or TCA cycle must be transported into the matrix space of mitochondria. The transport of individual molecule species is catalyzed by their specific transport proteins. These transporters show structural similarities with each other and are thought to have been formed from a common ancestral gene; and, hence, they are referred to as members of the mitochondrial solute carrier family, SLC25a (for reviews, see Palmieri, 2013; Gutiérrez-Aguilar and Baines, 2013; Wohlrab, 2009; Arco and Satrústegui, 2005; Kunji, 2004).

Most carrier proteins have been conserved in both yeast and mammals. As manipulation of the yeast genome is much easier than that of the mammalian one, yeast would seem to be very useful for structure/function studies on mammalian mitochondrial solute carriers. Actually, numbers of yeast mitochondrial carriers such as Sam5p, Flx1p, Oac1p, Leu5p, Rim2p, Ort1p, and Crc1p have been successfully complemented by the human orthologs SLC25A26 (Kishita et al., 2015), SLC25A32 (Schiff et al., 2016), SLC25A34 (Marobbio et al., 2008), SLC25A42 (Fiermonte et al., 2009), SLC25A33 and SLC25A36 (Di Noia et al., 2014), SLC25A15 (Ersoy Tunalı et al., 2014; Marobbio et al., 2015), and SLC25A20 (IJlst et al., 2001). However, complementation of yeast mitochondrial carriers by their mammalian ortholog is not always

Abbreviations: PiC, mitochondrial phosphate carrier; hPiC, PiC of human; rPiC, PiC of rat; yPiC, PiC of yeast; ΔNrPiC, PiC lacking its presequence.

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successfully achieved. In the case of the mitochondrial ADP/ATP carrier, introduction of the expression vector of the native bovine carrier into yeast cells lacking a functional ADP/ATP carrier gene was not effective in rescuing them from their defect in ATP synthesis, indicating that the native bovine carrier cannot be functionally expressed in yeast mitochondria (Hashimoto et al., 1999). As the yeast carrier has a longer N-terminal sequence than the bovine one, we examined earlier whether it would be possible to express a chimeric bovine carrier whose N-terminal sequence had been substituted with the corresponding region of the yeast carrier and found that this was the case (Hashimoto et al., 1999). Not only the bovine carrier but also the human one could be functionally expressed in yeast cells as chimeric proteins (Hatanaka et al., 2001).

In the case of the mitochondrial phosphate carrier (PiC), the mammalian one, but not the yeast one, is synthesized with a presequence, which is cleaved at the mitochondria (Runswick et al., 1987; Ferreira et al., 1989; Dolce et al., 1994). Furthermore, an earlier study clearly demonstrated that deletion of the presequence of the mammalian mitochondrial phosphate carrier facilitates the import of the carrier into yeast mitochondria (Zara et al., 1992). However, the more intriguing question of whether or not the mammalian phosphate carrier can be expressed in a functional form in yeast mitochondria had not been addressed. Therefore, in the present study we examined whether the mammalian mitochondrial phosphate carrier could be functionally expressed in yeast cells.

2. Materials and methods

2.1. Materials

The haploid strain of *Saccharomyces cerevisiae* W303—1B (MATα *ade2–1 leu2–3, 112 his3–22,15 trp1–1 ura3–1 can1–100*) was used as the wild type (Hashimoto et al., 1999). The single- and multi-copy type expression vectors in yeast cells were prepared by introducing the promoter region of the yeast type 2 ADP/ATP carrier gene into pRS314 and pYO326, respectively, as described previously (Hashimoto et al., 1999). Antibody against the FLAG tag (code F7425-2MG) was purchased from Sigma-Aldrich.

2.2. Preparation of a yeast strain lacking its MIR1 gene (mir1Δ)

Disruption of the *MIR1* gene encoding the mitochondrial phosphate carrier in the W303-1B strain was achieved by homologous recombination (*mir1::HIS3*). The structure of the targeting construct used for disruption of the *MIR1* gene is shown in Supplementary Fig. S1a. This construct was prepared by PCR. Briefly, the first PCR was carried out by using GE2857 and GE2859 as primers, and pRS313 as a template, as shown in Supplementary Fig. S1b. The nucleotide sequences of one-third of the 3′ side of these two primers corresponded to those at the 5′ and 3′ regions of the *HIS3* gene, respectively, in the pRS313; and the nucleotide sequences of their remaining regions corresponded to those at the 5′ and 3′ regions of the *MIR1* gene of the yeast genome. The second PCR was carried out by using GE2858 and 2860 as primers, and the PCR product of the first reaction as a template. Actual nucleotide sequences of individual primers are shown in Supplementary Fig. S1c. The nucleotide sequences of GE2857 and GE2859, shown in green, are those that annealed to the *HIS3* gene; and the boxed nucleotide sequences represent overlapped sequences between primers GE2857 and GE2858, and those between primers GE2859 and GE2860. The resultant PCR product was gel purified and used for transformation.

2.3. Preparation of cDNA and expression vectors of yeast, rat, and human mitochondrial phosphate carriers (abbreviated as yPiC, rPiC, and hPiC, respectively), and their mutants

The cDNA fragments encoding yPiC, rPiC, and hPiC were prepared by RT-PCR or PCR. Strategies for PCR, template DNA, and nucleotide primers used for preparation of these cDNA fragments are summarized in Supplementary Table SI. These cDNA fragments were subcloned into single copy-type (pRS314) or multi copy-type (pYO326) expression vectors having the promoter region of the yeast type 2 ADP/ATP carrier gene (referred to as pRS314/yA2P and pYO326/yA2P, respectively). The methods used for preparation of expression vectors of PiCs having a C-terminal FLAG tag are summarized in Supplementary Fig. S2.

2.4. Observation of cell growth

To examine the growth-defect phenotype, we streaked a yeast cell suspension on agar plates containing 1% yeast extract, 2% bactopeptone, and 2% agar supplemented with either 2% glucose (YPD) or 3% glycerol (YPGly) as a carbon source. After incubation at 30 °C for 2 or 5 days, the cell growth was assessed photographically.

2.5. Preparation of mitochondrial fraction from cultured yeast cells and Western blotting

For preparation of mitochondria, yeast cells were cultured in a liquid medium containing 1% yeast extract, 2% bactopeptone, and 2% galactose at 30 °C. The mitochondria fraction was prepared as described previously (Yamada et al., 2009a).

Western blotting was performed as described earlier (Yamada et al., 2009a; Yamada et al., 2009b). Samples of 20 μg (for detection of FLAG-tagged PiC) or 5 μg (for Por1p) aliquots of mitochondrial fractions were subjected to SDS-PAGE and subsequent Western blotting, and PiCs having the FLAG tag were detected by use of anti-FLAG antibody. Yeast Por1p (mitochondrial porin, also referred to as voltage-dependent anion channel) was also detected as a control by using anti-Por1p antibody, which was raised in a rabbit immunized with a synthetic peptide with the amino acid sequence of IVGGAEFGYDISAGSISRYC (amino acids 147–165 of yeast Por1p (NM_001182894) plus a C-terminal Cys residue for conjugation with hemocyanin) as the immunogen.

To ascertain the proper preparation of the mitochondrial fraction from yeast cells, we subjected a whole cell lysate and mitochondrial fraction to Western blotting by using antibodies against FLAG tag, Por1p, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Results obtained from *mir1Δ* and that expressing yPiC are shown in Supplementary Fig. S3.

2.6. Identification of amino acids important for functional expression of rPiC by PCR-based random mutation and gap-repair cloning

The outline of the PCR-based random mutation and gap-repair cloning procedure is depicted in Supplementary Fig. S4.

The randomly mutated cDNA fragments corresponding to the open reading frame of rat PiC lacking its N-terminal presequence (ΔNrPiC-A) were obtained by PCR using *Taq* DNA polymerase, because it is well known that mutation of DNA will occur at a certain frequency during ordinary PCR using this polymerase (Barnes, 1994). The amplification conditions used (25 μl) consisted of 0.2 ng template DNA (pYO326/yA2p/ΔNrPiC-A), 25 pmol each of primers (GE2046, 5′-GTAATACGACTCACTATAG and GE2047, 5′-TACAAGTCAAAGGAGCCCC), 1× HY buffer, 200 μM dNTP, and 1.25 units of *Taq* DNA polymerase (Greiner, code 986007). The mixture was heated at 95 °C for 5 min and then subjected to a chain reaction of 35 cycles of heating at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 15 min.

The resulting amplification mixture was mixed with 10 μg aliquots of the expression vector of ΔNrPiC (rPiC lacking its N-terminal presequence) digested with *Bam*HI and *Bgl*II. Then, the yeast cells lacking their *MIR1* gene were transformed with the above-mentioned mixture, and spread on an agarose plate containing minimum medium with glycerol as a carbon source but lacking uracil and histidine. After