Significant Expression of Functional Human Type 1 Mitochondrial ADP/ATP Carrier in Yeast Mitochondria

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As a first step to characterize the unknown functional properties of the human mitochondrial ADP/ATP carrier (AAC), we tried to express human type 1 AAC (hAAC1) in *Saccharomyces cerevisiae*. Expression of hAAC1 in yeast mitochondrial membrane was very low, although its transcript level was high. Its expression was improved greatly by replacement of its *N*-terminal region with the corresponding region of yeast type 2 AAC (yAAC2), as observed with the bovine type 1 AAC (bAAC1). This chimeric hAAC1 showed similar ADP transport activity to that of chimeric bAAC1, corresponding to the transport activity of bAAC1 in bovine heart mitochondria. These results suggested that the *N*-terminal region of yAAC2 is important for expression of the mammalian carriers in yeast mitochondria. Using the present expression system, studies on the functional properties of the human AAC isoforms in relation to their structures are now possible.

Key words human ADP/ATP carrier; mitochondria; *Saccharomyces cerevisiae*; oxidative phosphorylation; functional complementation

The 30 kDa ADP/ATP carrier (AAC or ANT) in the mitochondrial inner membrane mediates the exchange transport of ADP and ATP from both the cytosolic and matrix sides, and is a member of the mitochondrial solute carrier family.¹⁾ As this carrier is essential for oxidative phosphorylation, it has received considerable attention.^{2–5)}

In *Saccharomyces cerevisiae*, three different AAC isoforms, yAAC1, yAAC2 and yAAC3 are expressed depending on physiological conditions.^{6–9)} Of these, yAAC2 is mainly responsible for transport of ADP and ATP to support yeast growth with a non-fermentable carbon source,⁶⁾ whereas yAAC3 is essential for growth under anaerobic conditions,^{8,9)} and yAAC1 is expressed slightly only under aerobic conditions.⁶⁾ Hence, yAAC2 is the representative yeast AAC isoform, and its structural features in relation to its function have been studied extensively by site-directed mutagenesis.^{4,5)} Various AAC isoforms are also expressed in mammalian mitochondrial membrane in tissue-specific manners.^{4,10)} Of the mammalian AACs, structural and functional characterizations have been well performed with bovine type 1 AAC (bAAC1)^{2–4)} due to its abundant expression in heart mitochondria, corresponding to 10% of the total amounts of proteins in the inner mitochondrial membrane.^{10–12)}

In humans, three isoforms of AAC, ANT1, ANT2 and ANT3 are encoded by three different genes.^{13,14)} In contrast to the bovine AAC, expression of human AAC isoforms has been well studied under various physiological and pathological conditions.^{15–19)} The human ANT1, referred to as hAAC1 in this paper, is predominantly expressed in heart, skeletal muscle and brain.^{15,16)} ANT2 is expressed in a broad range of tissues, particularly rapidly proliferating tissues such as liver, kidney, fibroblasts and lymphocytes, and ANT3 is expressed broadly, but its expression is low in all tissues.^{15,16)} The tissue-specific transcription of the human AAC isoforms is thought to be related to energy requirements associated especially with the state of cellular differentiation.¹⁶⁾ Furthermore, total amounts of AACs expressed in heart mitochondria of patients with cardiomyopathy, in which the ADP/ATP transport capacity was decreased, were reported to

be increased accompanied by a shift in the population of AAC isoforms as determined by their transcript levels: increase and decrease in the amounts of hAAC1 and hAAC2, respectively, without change in the content of hAAC3.^{17–19)} Thus, the physiological and the pathological significance of human AAC isoforms are thought to be associated with their functional properties.

To understand the physiological and pathological roles of human AAC isoforms on a molecular level, it is necessary to characterize their functional properties. However, there is no study on the functional properties of the human AAC isoforms, because isolation of the human carriers is difficult. In addition, their bacterial expression will be difficult, like the bovine carrier.²⁰⁾ Furthermore, it is not evident whether the structural and functional features of bAAC1 are comparable with those of human AAC. As a first step in studying the functional characterization of the human AAC isoforms, we tried to express the most representative isoform, hAAC1 in the mitochondrial membrane of *S. cerevisiae*.

Recently, we succeeded in functional expression of bAAC1 in *S. cerevisiae*.²¹⁾ Although bAAC1 is expressed slightly in the yeast mitochondrial membrane, its expression was elevated to that of yAAC2 by replacement of its *N*-terminal region extending into the cytosol by the corresponding region of yAAC2, and the transport activity of the expressed chimeric bAAC1 was essentially identical with that of native bAAC1 in bovine heart mitochondria.²¹⁾ If the expression of hAAC1 in yeast mitochondria is achieved, this system will be useful for studies on the structure-based function of human AACs.

MATERIALS AND METHODS

Materials and Reagents The haploid strain of *S. cerevisiae*, W303-1B (MAT α *ade2-1 leu2-3,112 his3-22,15 trp1-1 ura3-1 can1-100*)⁶⁾ was a gift from Dr. S. Shimizu (Osaka University). The AAC-disrupted strain of WB-12 (MAT α *ade2-1 trp1-1 ura3-1 can1-100 aac1::LEU2 aac2::HIS3*) and the yeast shuttle vector pRS314-YA2P were prepared as

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described.²¹⁾ Carboxyatractyloside (CATR) was obtained from Sigma (St. Louis, MO, U.S.A.), and bongkreikic acid (BKA) was a gift from Prof. Duine (Delft University of Technology). Other materials and reagents were of the high-grade commercially available.

Preparation of DNA Fragments Encoding Various AACs A DNA fragment corresponding to the open reading frame (ORF) encoding hAAC1 was prepared by polymerase chain reaction (PCR). A template for PCR was prepared by reverse transcription of mRNA of human heart (Clontech). The amplimers MB67 (5'-CGAGCTGTCcatATGGTGATCA, nucleotides -12 to 11,¹³⁾ in which the underlined and small case letters represent the created restriction sites and altered nucleotides, respectively) and MB68 (5'-CCACA-CAATGGATC_cGTGAAC, nucleotides 965 to 945¹³⁾) were used for preparation of a DNA fragment encoding hAAC1 which has restriction sites of *NdeI* and *BamHI* in the 5'- and 3'-ends, respectively. DNAs corresponding to the ORFs encoding yAAC2 and bAAC1 were similarly prepared.²¹⁾

For preparation of chimeric hAAC1 in which the N-terminal region of the hAAC1 was replaced by the corresponding region of yAAC2, a restriction site of *NaeI* in the DNA fragment encoding the hAAC1 was created at the corresponding *NaeI* site in a DNA encoding bAAC1²¹⁾ by PCR using a DNA encoding the hAAC1 as a template, and the amplimers MB93 (5'-GCCGGcGGCGTCGCCGCTGCCGTC, nucleotides 40 to 63¹³⁾) and MB68. A DNA fragment encoding the N-terminal region of yAAC2 was also prepared by PCR using a DNA encoding yAAC2 as a template and the amplimers HT499 (5'-ATAc_{at}ATGTCTTCTAACGCC, nucleotides -6 to 16) and HT544 (5'-GCACTGACACCgCCg_{gc}TAAGAA, nucleotides 101 to 79).²¹⁾ A DNA fragment encoding chimeric hAAC1 (y2NhAAC1) was prepared by ligation of the 87 bp *NdeI*-*NaeI* DNA fragment encoding the N-terminal region of the yAAC2 into the 5'-terminus of DNA encoding the hAAC1 which did not contain its N-terminal region through the *NaeI* site. A DNA fragment encoding chimeric bAAC1 (y2NbAAC1) was similarly prepared.²¹⁾ The *NdeI*-*BamHI* fragments encoding these AACs and chimeric AACs prepared were subcloned into the *NdeI* and *BamHI* sites of pRS314-YA2P for transformation of the AAC-disrupted yeast strain WB-12.²¹⁾ The growths of these transformed yeast cells were examined as described.²¹⁾

Western and Northern Blotting Yeast mitochondria were prepared as described previously.²¹⁾ SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel was performed by the method of Laemmli.²²⁾ The amounts of carriers expressed in yeast mitochondria were determined by Western blotting using antiserum against a synthetic peptide having the yAAC2 sequence (Asn⁵³-Lys⁷⁵), or the hAAC1 sequence (His³⁹-Ile⁶⁰), which completely corresponds to that of the bAAC1.¹³⁾ Northern blotting was performed as described.²¹⁾

Measurement of ADP Transport Activity The ADP transport activity of yeast mitochondria was determined essentially as described.²³⁾ Mitochondria (1 mg of protein/ml) suspended in medium consisting of 250 mM sucrose, 1 μ g/ml oligomycin, 0.2 mM EDTA-2Na and 10 mM Tris-HCl buffer (pH 7.4) in a total volume of 200 μ l were allowed to stand for 5 min at 0 °C. The ADP transport was started by addition of a final concentration of 100 μ M [¹⁴C]ADP (specific radioactiv-

Fig. 1. Growth of Yeast Cells on Glycerol Plate

The transformants were inoculated on a glycerol plate and their growth was examined after 1 week at 30 °C. WT, wild-type W303-1B; WB-12, WT in which the yAAC1 and yAAC2 genes were disrupted; yAAC2, hAAC1, y2NhAAC1, bAAC1 and y2NbAAC1, WB-12 cells transformed with vectors with inserted DNA fragments encoding these AACs; vector, WB-12 transformed with vector without DNA fragments encoding AACs.

ity, 37 kBq/ μ mol, and after the desired incubation period, transport was terminated with 20 μ M CATR and 5 μ M BKA. The amount of [¹⁴C]ADP incorporated was determined from its radioactivity in an Aloka liquid scintillation counter LSC-3500. The initial rate of ADP uptake was determined as reported.²⁴⁾

RESULTS

Expression of Native hAAC1 in the Yeast Mitochondrial Membrane First, we examined whether the native human type 1 ADP/ATP carrier (hAAC1) could be functionally expressed in yeast mitochondria. For this, we prepared a transformant of the AAC-disrupted strain, WB-12, in which the intrinsic yAAC1 and yAAC2 genes of the wild-type strain of *S. cerevisiae* W303-1B were disrupted. The DNA fragment encoding hAAC1 was introduced into WB-12 cells by a single-copy plasmid pRS314-YA2P having the promoter region of the yAAC2 gene as a vector, and growth was examined on a plate containing 3% glycerol as a non-fermentable carbon source. Similarly, DNA fragments of the yAAC2 and bAAC1 were prepared, introduced into WB-12 cells, and growth of the yeast transformants were examined. As shown in Fig. 1, the AAC-disrupted strain WB-12 did not grow at all on a glycerol plate due to inefficient oxidative phosphorylation activity. The yeast transformants of hAAC1 and bAAC1 appeared not to grow, but the transformant of yAAC2 grew well to an extent similar to that of the wild-type strain W303-1B. The growth doubling times of the wild-type strain and the transformant of yAAC2 in liquid glycerol medium were both about 4 h.

Next, we isolated mitochondria from yeast cells and subjected the mitochondrial proteins to SDS-PAGE and Western blotting using antiserum against a synthetic peptide of the yAAC2 sequence (Asn⁵³-Lys⁷⁵) and that of the hAAC1 sequence (His³⁹-Ile⁶⁰), which is completely conserved in the corresponding region of the bAAC1.¹³⁾ As shown in Fig. 2, the 34 kDa bands of yAAC2 from the wild-type strain and yAAC2 transformant were immunostained to similar extents with the anti-peptide antibody against yAAC2. However, the intensities of the 30 kDa bands of hAAC1 and bAAC1 immunostained with the antibody against hAAC1 were very low. The levels of expression in yeast mitochondrial mem-

Fig. 2. SDS-PAGE and Western Blotting of Mitochondrial Proteins from Yeast Cells

Isolated mitochondrial proteins were subjected to SDS-PAGE. Samples of 10 and 5 μ g protein were detected by staining with Coomassie Brilliant Blue R-250 (A), and by immunostaining with anti-peptide antibody against yAAC2 (Asn⁵³–Lys⁷⁵) (B) and against hAAC1 (His³⁹–Ile⁶⁰) (C). WT, WB-12, yAAC2, hAAC1, y2NhAAC1, bAAC1 and y2NbAAC1 are same as for Fig. 1. The result with 2 μ g bovine heart mitochondria (BHM) is shown as a control.

Table 1. Amounts of AAC Expressed in Yeast Mitochondrial Membranes and ADP Transport Rates across Mitochondrial Membranes

AAC ^{a)}	Amount of AAC ^{b)} (nmol AAC/mg proteins)	v_{ADP} ^{c)} (mol ADP/mol AAC/min)
WB-12 ^{d)}	n.d. ^{e)}	n.d. ^{e)}
yAAC2	0.72 $\pm$ 0.05	87.5 $\pm$ 10.6
hAAC1	0.03 $\pm$ 0.01	n.d. ^{e)}
y2NhAAC1	0.64 $\pm$ 0.07	19.8 $\pm$ 4.1
bAAC1	0.005 $\pm$ 0.001	n.d. ^{e)}
y2NbAAC1	0.60 $\pm$ 0.07	20.8 $\pm$ 7.9
BHM ^{f)}	2.65 $\pm$ 0.47 ^{g)}	24.6 $\pm$ 5.7

a) Yeast, human and bovine AACs expressed in yeast transformants. b) Amount of AAC expressed in yeast mitochondria. Values are means $\pm$ S.D. for three separate experiments. c) Initial velocity ($\pm$ S.D.) of ADP uptake at 0 $^{\circ}$ C and pH 7.4 by yeast mitochondria in three separate experiments. d) Total possible AACs in disruptant WB-12. e) Not determined due to low amount of AAC. f) Bovine heart mitochondria. g) From ref. 21.

branes were determined from the intensities of the immunostained bands of these carriers,²¹⁾ as summarized in Table 1.

Effect of the N-Terminal Region of yAAC2 on the Expression of hAAC1 in Yeast Mitochondria Recently, we found that the slight expression of the bAAC1 in yeast mitochondria was significantly enhanced by replacement of its N-terminal region which is thought to extrude in the cytosolic side by the corresponding region of yeast carriers.²¹⁾ Therefore, we next examined the effect of replacement of the N-terminal region of hAAC1 by that of yAAC2 on its expression in the yeast mitochondrial membrane. We prepared DNA fragments encoding chimeric hAAC1 and bAAC1, in which the first 11 residues of their N-terminal segments were replaced by the corresponding 26 amino acid residues of yAAC2, using the restriction site of *NaeI*. These chimeric carriers are referred to as y2NhAAC1 and y2NbAAC1, respectively. The DNA fragments thus prepared were introduced into the disruptant WB-12. As shown in Fig. 1, the transformants of y2NhAAC1 and y2NbAAC1 grew well on a glycerol plate. Their doubling times in liquid glycerol medium were both about 8 h, being twice that of the transformant of yAAC2. Hence, the oxidative phosphorylation ability of the AAC-disrupted WB-12 cells was well complemented by introduction of y2NhAAC1 or y2NbAAC1.

As shown in Fig. 2, SDS-PAGE and Western blotting

Fig. 3. Steady-State Transcript Levels of AACs in Yeast Cells

Samples of RNA (10 μ g) were separated by electrophoresis in formaldehyde containing gel and transferred to nitrocellulose membranes, then they were hybridized with an *NdeI*–*NaeI* DNA fragment encoding yAAC2 (A), and *NaeI*–*BamHI* fragments encoding hAAC1 (B) and bAAC1 (C). Abbreviations of the carriers are same as for Fig. 1. The amounts of RNA samples were confirmed from the intensities of 28S rRNA stained with methylene blue on Northern blotting with the same amount of RNA samples (D). Values under lanes are amounts of mRNA relative to that of yAAC2 determined from signal intensities of autoradiograms. Values for human and bovine carriers were determined from the signal intensities hybridized with the corresponding probes in panels (B) and (C), respectively²¹⁾.

showed that 34 kDa y2NhAAC1 and y2NbAAC1 were significantly expressed in yeast mitochondrial membranes, and their amounts were determined from the intensities of immunostained bands,²¹⁾ as listed in Table 1. Chimeric hAAC1 and bAAC1 were expressed to similar extents to that of yAAC2. Like bAAC1, replacement of the N-terminal region of hAAC1 by the corresponding region of yAAC2 elevated the expression to more than 20 times that of native hAAC1.

Transport Activity of Chimeric hAAC1 in Yeast Mitochondria Next, we examined the ADP transport activity of mitochondria of various transformants. The ADP transport in mitochondria of the transformant of y2NhAAC1 was inhibited by transport inhibitors of CATR and BKA. The initial transport velocity of ADP through the mitochondrial inner membranes (v_{ADP}) was determined at 0 $^{\circ}$ C and pH 7.4, and the results are summarized in Table 1. The v_{ADP} value of yAAC2 in yeast mitochondria of the transformant was similar to that expressed in the wild-type W303-1B,²¹⁾ showing that the transport of ADP/ATP in the transformant was as operative as in the wild-type. The v_{ADP} values of native hAAC1 and bAAC1 could not be determined due to their low expression in yeast mitochondria. In contrast, the value of y2NhAAC1 was determined to be almost the same as that of y2NbAAC1, the v_{ADP} of which was essentially identical with that of native bAAC1 in bovine heart mitochondria (Table 1).

Steady-State Transcript Levels of Native and Chimeric AACs in Yeast Cells We next performed Northern blot analyses of the steady-state transcript levels of the native and chimeric carriers in yeast cells. To determine those levels, we prepared an *NdeI*–*NaeI* fragment of the yAAC2 and *NaeI*–*BamHI* fragments of the hAAC1 and bAAC1 as specific probes. As shown in Fig. 3, the probe of yAAC2 hybridized with RNA samples of yAAC2, y2NhAAC1 and y2NbAAC1, but not with those of native hAAC1 or bAAC1. The hybridized bands with probes of hAAC1 and bAAC1 were observed with RNA samples of the corresponding native and

chimeric carriers. In addition, cross-hybridizations with both probes were observed with RNA samples of human and bovine carriers due to their highly conserved sequences. As the hybridization band intensities of various RNA samples with different probes can be normalized in terms of those with a common probe,²¹⁾ the transcript levels of all the carriers examined were compared from the radioactivities of the hybridized bands. The values thus determined are shown at the bottom of autoradiograms (Fig. 3). It is noteworthy that the transcript levels of hAAC1 were highest of all the carriers, showing that slight expression of native hAAC1 was not due to its low transcript level.

DISCUSSION

The physiological and pathological significances of the AAC isoforms expressed in a tissue-specific manner are thought to be related to difference in their functional properties.^{15–19)} Previous studies on the functional properties of the yeast AAC isoforms showed that there are no substantial differences among them in the number of binding sites and affinities for ADP and ATP, whereas the transport activity of yAAC1 is about 40% of that of yAAC2.^{25,26)} Such studies should be especially important for human AAC isoforms. However, no relevant studies have yet been performed, possibly due to difficulty in their isolation from human mitochondria, and in their expression in bacteria and yeast cells as in bAAC1.^{20,21)} Hence, as a first step in study of the functional properties of human AAC isoforms, we tried to express human type 1 AAC (hAAC1) in AAC-disrupted *S. cerevisiae*. This isoform is suggested to be expressed most abundantly in human heart mitochondrial membrane as determined from its mRNA level.^{15,16)}

In this study, we found that native hAAC1 was expressed in the yeast mitochondrial membrane, although its expression was very low. However, this low expression was significantly improved by replacement of its N-terminal region extending into the cytosol by the corresponding region of yAAC2, and the oxidative phosphorylation activity of the AAC-disrupted yeast strain WB-12 was well complemented by expression of the chimeric hAAC1. This finding is consistent with our previous results on bAAC1.²¹⁾ As the transcript level of the native hAAC1 was higher than that of the chimeric hAAC1 and that of the native bAAC1 was comparable with that of its chimeric carrier, the low expression of the hAAC1 as well as bAAC1 is not due to its inefficient transcription or low stability of mRNA, but to problems after transcription such as translation, stability of the expressed hAAC1 and import into the yeast mitochondrial membrane.

No proteolytic processing takes place after localization of the AAC into mitochondria, and the targeting signal of the AAC is thought to be contained in its mature form.^{27–29)} Previous results showed that the 72nd–111th amino acid residues in the middle part of yAAC1²⁷⁾ and each of the three homologous domains of *N. crassa* AAC contains information for mitochondrial targeting.^{28,29)} However, significant elevation of the expressions of hAAC1 and bAAC1 by replacement of their N-terminal regions suggested that the N-terminal region of yAAC2 is also important for expression of the carrier in yeast mitochondrial membranes, and possibly for its efficient localization there.²¹⁾

Furthermore, we found that the expressed chimeric hAAC1 was sensitive to the transport inhibitors CATR and BKA as in bAAC1, and the v_{ADP} value of chimeric hAAC1 was comparable with that of chimeric bAAC1. The v_{ADP} of chimeric bAAC1 in yeast mitochondrial membranes was essentially the same as that of native bAAC1 in bovine heart mitochondria, suggesting that the chimeric hAAC1 expressed in yeast mitochondria functions like native hAAC1 in human mitochondria, and that the hAAC1 in human mitochondria shows similar transport activity to that of the bAAC1. In addition, 12 amino acid residues that are not conserved in human and bovine carriers¹³⁾ were probably not associated with the transport activity. Furthermore, the N-terminal region of the AACs extending into the cytosol was suggested not to be important for their transport activity.

In this study, expression of mammalian AACs was achieved under control of the intrinsic yAAC2 gene promoter but not an inducible promoter such as gal10/cyc1 promoter in the AAC-disrupted yeast cells. Hence, the present expression system is useful to examine whether the introduced AAC is functionally expressed in the yeast mitochondrial membrane from the growth of the transformants in a non-fermentable carbon source. In addition, the use of single-copy plasmid is advantageous for studies on revertants. To date, amino acid sequences of various mammalian AACs have been reported such as those with mouse and rat AAC isoforms as well as human and bovine isoforms.⁴⁾ Their homology is as high as 87–99% and their N-terminal regions thought to extend into the cytosol consist of 11 amino acid residues. Therefore, functional expression of these mammalian AACs will be available in yeast mitochondrial membranes by replacement of their N-terminal region with the corresponding region having 26 amino acid residues of the yAAC2.

This is the first report on the functional expression of human AAC possibly retaining its native transport activity. Studies on the molecular mechanism of transport function as well as physiological and pathological roles of human AAC are now possible using the chimeric carrier expressed in yeast mitochondrial membranes.

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