The *Drosophila* gene 2A5 complements the defect in mitochondrial F_1-ATPase assembly in yeast lacking the molecular chaperone Atp11p

Zhen-Guo Wang[a], Karl J. Schmid[b], Sharon H. Ackerman[a,*]

[a] *Department of Surgery and Department of Biochemistry and Molecular Biology, Wayne State University, School of Medicine, Detroit, MI 48201, USA*

[b] *Section of Genetics and Development, Cornell University, Ithaca, NY 14853, USA*

Received 15 April 1999

Abstract Assembly of mitochondrial F_1-ATPase in *Saccharomyces cerevisiae* requires the molecular chaperone, Atp11p. Database searches have identified protein sequences from *Schizosaccharomyces pombe* and two species of *Drosophila* that are homologous to *S. cerevisiae* Atp11p. A cDNA encoding the putative Atp11p from *Drosophila yakuba* was shown to complement the respiratory deficient phenotype of yeast harboring an *atp11::HIS3* disruption allele. Furthermore, the product of this *Drosophila* gene was shown to interact with the *S. cerevisiae* F_1 β subunit in the yeast two-hybrid assay. These results indicate that Atp11p function is conserved in higher eukaryotes.

Key words: Yeast; Atp11p; F_1-ATPase; *Drosophila*

1. Introduction

The ATP synthase of mitochondrial, chloroplast, and bacterial membranes catalyzes the synthesis of ATP coupled to respiration [1,2]. The enzyme is composed of an integral membrane component called F_o, and a peripheral catalytic moiety called F_1. Membrane-bound F_1 catalyzes both ATP synthesis and ATP hydrolysis in reactions that are coupled to proton translocation through the F_o. Soluble F_1 (F_1-ATPase) functions solely as an ATP hydrolase.

F_1 contains five different types of subunits in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\varepsilon$ [1,2]. Crystal structures have been solved for bovine [3] and rat liver [4] mitochondrial F_1. The α and β subunits alternate in a hexamer that surrounds the rod-shaped γ subunit [3,4]. Previous work with respiratory deficient mutants of *Saccharomyces cerevisiae* has indicated that formation of the F_1 α/β hexamer requires the products of the *ATP11* [5] and *ATP12* genes [6]. In *atp11* or *atp12* mutants the F_1 oligomer is not formed; instead such strains accumulate the α and β subunits in large protein aggregates inside mitochondria [7]. Evidence has been obtained for direct binding between Atp11p and the F_1 β subunit, and between Atp12p and the F_1 α subunit (manuscripts in preparation). We propose that such binding interactions maintain the F_1 subunits in soluble form during enzyme assembly. On the basis that the actions of Atp11p and Atp12p appear to be restricted to F_1 assembly [7], these proteins can be considered 'F_1-specific' molecular chaperones.

In this communication we report evidence that the action of Atp11p in mitochondrial F_1-ATPase assembly has been conserved in evolution. Database searches identified protein products deduced from genomic or cDNA sequences from *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and *Drosophila yakuba* that are homologous with Atp11p from budding yeast. Additional studies provide strong evidence that the fly protein sequences define true Atp11p homologs. First, the relevant cDNA from *D. yakuba* (called 2A5) rescued the respiratory defect of a yeast *atp11* mutant. Second, the product of the 2A5 gene was shown to interact with the yeast F_1 β subunit in the two-hybrid screen.

2. Materials and methods

2.1. Plasmids and strains

Recombinant plasmids and *S. cerevisiae* strains used in this study are described in Table 1. *Escherichia coli* RR1 (*pro*A *leu*B *lac*Y *gal*K *xyl*-5 *mtl*-1 *ara*-14 *rps*L *sup*E *hsd*S λ$^-$) was the host bacterial strain for the recombinant plasmids. To construct the *CEN* plasmid pCUP2A5/CEN316, the yeast *CUP1* promoter was excised as a 430 bp *Bam*HI-*Eco*RI fragment from pND164 [8], and the *D. yakuba* 2A5 cDNA was prepared as a 1.1 kb *Eco*RI-*Xho*I fragment. Both DNA fragments were then ligated in concert with *Bam*HI,*Xho*I-cut pRS316 [9]. To make the 2μ plasmid (pCUP2A5/YEp) that carries *D. yakuba* 2A5 DNA under transcriptional control of the *CUP1* promoter, a 1.5 kb *Bam*HI-*Kpn*I fragment was subcloned from pCUP2A5/CEN316 into the *Bam*HI and *Kpn*I sites of YEp352 [10]. To make pAS2-1/2A5(33–278), an 800 bp fragment was prepared from pCUP2A5/YEp as a *Hga*I(blunted), *Kpn*I fragment and ligated to the *Eco*RI(blunted) and *Kpn*I sites of pTrc99A (Pharmacia). An 850 bp *Nco*I-*Bam*HI fragment was then prepared from this intermediate plasmid and ligated to the *Nco*I and *Bam*HI sites of pAS2-1 (Clontech). To make pACT2/ATP2(36–511), *ATP2* DNA was prepared as a 1.5 kb *Pvu*II-*Xho*I fragment and ligated with pACT2 (Clontech) that was prepared as a *Xma*I(blunted), *Xho*I fragment.

2.2. Media

Yeast was grown in the following media: YPD (2% glucose, 2% peptone, 1% yeast extract), YPGal (2% galactose, 2% peptone, 1% yeast extract), YEPG (2% glycerol, 2% ethanol, 2% peptone, 1% yeast extract), WO (2% glucose, 0.67% yeast nitrogen base without amino acids (Difco)), SD/−his,trp,leu (2% glucose, 0.67% yeast nitrogen base without amino acids, supplemented with all essential amino acids and nucleotides except histidine, tryptophan, and leucine). Amino acids and other growth requirements were added at a final concentration of 20–150 μg/ml. *E. coli* bearing recombinant plasmids were grown in LB medium [11] supplemented with 40 μg/ml ampicillin. The solid media contained 2% agar in addition to the components described above.

2.3. Yeast two-hybrid assay

The yeast two-hybrid assay [12] employed yeast vectors (pACT2 and pAS2-1) and host strain Y190 (described above) that were supplied in the MATCHMAKER Two-Hybrid System 2 from Clontech. Yeast were grown in SD/−his,trp,leu medium. Expression from the *lacZ* reporter gene was determined using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) as a chromogenic substrate for β-ga-

*Corresponding author. Fax: (1) (313) 577-7642.
E-mail: sackerm@med.wayne.edu

PII: S0014-5793(99)00676-6

lactosidase in the colony-lift filter assay described in the Clontech manual.

2.4. Miscellaneous procedures

Standard techniques were used for restriction endonuclease analysis of DNA, purification and ligation of DNA fragments, transformations of and recovery of plasmid DNA from *E. coli* [11]. Yeast transformations employed the LiAc procedure [13]. Preparation of yeast mitochondria and ATPase assays were done as described previously [5]. Protein concentrations were estimated by the method of Lowry et al. [14].

3. Results

3.1. Database matches with yeast Atp11p

Atp11p from *Saccharomyces cerevisiae* was used as the query sequence to search the protein sequence databases with the Blastp program [15]. This analysis identified homologous amino acid sequences from *S. pombe* (GenBank accession number Z95395; BLAST score, 143; $P = 1 \times 10^{-33}$), *D. melanogaster* (GenBank accession number AF005855; BLAST score, 80; $P = 3 \times 10^{-14}$), and *D. yakuba* (GenBank accession number AF005856; BLAST score, 78; $P = 1 \times 10^{-13}$). Alignment of *S. cerevisiae* Atp11p with the other three proteins shows 50% similarity (including identical and physico-chemically similar amino acids) and 14.6% identity, with similar regions distributed uniformly along the sequence (Fig. 1). A pairwise comparison of the overall similarities and identities between the four homologous sequences is shown in Table 2. The DNA sequence encoding the Atp11p homolog from *S. pombe* was deposited in the database as part of the *S. pombe* genome sequencing project[1]; no functional data have been reported for this gene product. The *D. melanogaster* and *D. yakuba* cDNA sequences were identified during a screen for fast evolving genes in *Drosophila* and designated with the gene name 2A5 [17].

The protein sequence of *S. cerevisiae* Atp11p shown in Fig. 1 includes a mitochondrial targeting sequence at the amino-terminus [5]; the first amino acid of the *mature* protein is predicted to be Glu-40 [18] (boxed residue in *S. cerevisiae* Atp11p sequence, Fig. 1). Previous studies with partially deleted forms of *S. cerevisiae* Atp11p provided information that the functional domain of the protein is located within the sequence bordered by Asp-112 (see arrow, Fig. 1) and Arg-183 (see asterisk, Fig. 1) [19]. Owing to the presence of proline-rich sequences in the vicinity of these two amino acids, we previously designated the region between Phe-120 and Asn-174 as the 'minimal' domain necessary for function (see black bar, Fig. 1), nestled between Pro-rich 'connector' sequences [19]. However, inspection of the multiple alignment shows that the distal of the two *S. cerevisiae* Pro-rich sequences (Pro-175 through Arg-183, see white bar in Fig. 1) is *78% identical* in all four proteins. This observation suggests that the proline-rich PXFXLPLPR sequence should be considered part of the functional domain of Atp11p, rather than a linker segment in the protein as formerly proposed.

3.2. Functional studies with Drosophila Atp11p

The full-length 2A5 cDNA from *D. yakuba* was subcloned

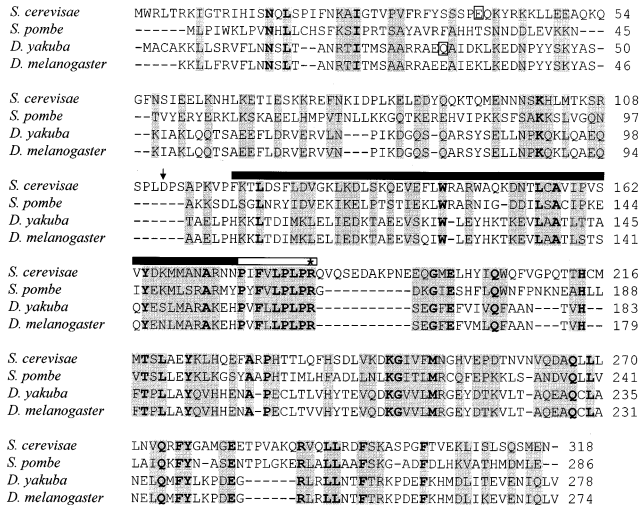

Fig. 1. Homologous Atp11p sequences. The amino acid sequence of Atp11p from *S. cerevisiae* was aligned with homologous sequences from *S. pombe*, *D. yakuba*, and *D. melanogaster* using the CLUSTALW program [16]. Shading indicates identical and physico-chemically similar amino acids; identical amino acids are further highlighted in bold. The boxed amino acids in the sequences from *S. cerevisiae* and *D. yakuba* are the approximated start sites for the mature proteins. The following symbols are used to indicate specific amino acids/spans of sequence in *S. cerevisiae* Atp11p that are discussed in the text: arrow, Asp-112; asterisk, Arg-183; black bar, Phe-120 through Asn-174; white bar, Pro-175 through Arg-183.

along with the yeast *CUP1* promoter into single and multicopy yeast episomal vectors yielding the plasmids pCUP2A5/CEN316 and pCUP2A5/YEp, respectively (Table 1). These plasmids were introduced, individually, into the respiratory deficient yeast strain W303ΔATP11 that is disrupted at the *ATP11* locus (Table 1). The transformed cells were then evaluated for growth on non-fermentable carbon sources (YEPG medium) as an indication of respiratory function. As shown in Fig. 2, W303ΔATP11 does not grow on YEPG plates incubated at 30°C for 3 days. However, within this time span, each of the plasmids carrying *D. yakuba* 2A5 cDNA conferred growth on the selective medium to the mutant strain (Fig. 2), although at rates significantly lower than the wild type (W303-1A) control. These results demonstrate a rescue of the yeast mutation by the *Drosophila* 2A5 gene. Mitochondrial ATPase activity measured for transformants of W303ΔATP11 that produce *D. yakuba* 2A5 from a single or multicopy vector is reported in Table 3. Under the conditions of this assay, wild type W303-1A typically shows 4–5 U/mg oligomycin sensitive ATPase activity [6]. The sub-optimal levels of mitochondrial ATPase activities detected in yeast that produce heterologous Atp11p from *D. yakuba* are in accord with the fact that these transformants grow much more slowly on YEPG medium versus the parental wild type strain (Fig. 2).

The yeast two-hybrid assay (see Section 2.3) was used to probe for binding interactions between the Atp11p homolog from *D. yakuba* and the yeast F_1 β subunit. The reporter strain (Y190) used in these experiments harbors *lacZ* under transcriptional control from Gal4p. The two-hybrid plasmid constructed with 2A5 cDNA (pAS2-1/2A5(33–278), Table 1) was designed to encode the *mature* portion of *D. yakuba* Atp11p fused to the distal end of the Gal4p DNA binding domain (bd) in the vector pAS2-1. Following the premise that mitochondrial leader peptides are rich in basic and hydroxyl-

[1] Wood, V., Barrell, B.G. and Rajandream, M.A. (Direct Submission 05/01/97), *Schizosaccharomyces pombe* chromosome I sequencing project, Sanger Centre, Hinxton Hall, Hinxton, Cambridge.

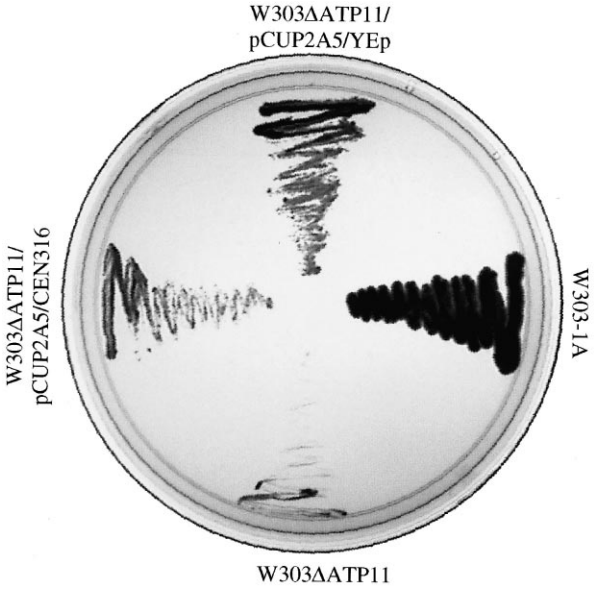

Fig. 2. Complementation of a yeast *atp11::HIS3* mutation with the 2A5 gene from *D. yakuba*. The following four *S. cerevisiae* strains were streaked on a YEPG plate and incubated for 3 days at 30°C: W303-1A, respiratory competent parental wild type yeast; W303ΔATP11, respiratory deficient mutant that harbors an *atp11::HIS3* allele; W303ΔATP11/pCUP2A5/CEN316 and W303ΔATP11/pCUP2A5/YEp; W303ΔATP11 transformed with single or multicopy plasmid that carries the 2A5 gene from *D. yakuba* under the control of the yeast *CUP1* promoter.

ated amino acids, and notably deficient in acidic residues [20], the mature Atp11p protein from *Drosophila* is predicted to begin in the vicinity of Gln-33 (boxed residue in *D. yakuba* Atp11p sequence, Fig. 1). For this reason the first 32 codons of the 2A5 sequence are omitted in pAS2-1/2A5(33–278). The other plasmid used in these experiments, pACT2/ATP2(36–511), encodes a protein chimera in which the Gal4p activation domain (ad) was fused distally to the mature portion of the yeast F_1 β subunit. The first amino acid in the mature yeast F_1 β subunit is Ala-34 (D. Mueller, The Chicago Medical School, personal communication); the mature form of the β subunit encoded in pACT2/ATP2(36–511) is missing the first two residues from the amino-terminus. Co-production of ad-(yeast F_1 β) and bd-(*Drosophila* Atp11p) fusion proteins in Y190 led to functional reconstitution of the Gal4p transcription factor. Gal4p induced activation of *lacZ* in the reporter strain was indicated by the fact that the cells showed blue color following lysis and exposure to X-gal on filter paper (data not shown). These results provide evidence for binding interactions between *D. yakuba* Atp11p and *S. cerevisiae* F_1 β subunit.

4. Discussion

Atp11p was identified almost 10 years ago to be a protein necessary for mitochondrial F_1 assembly in *S. cerevisiae* [7]. Here we report evidence for conservation of Atp11p action in other eukaryotes. Protein database analysis has revealed gene products from *S. pombe*, *Dr. yakuba*, and *D. melanogaster* that are homologous with *S. cerevisiae* Atp11p (Fig. 1). Moreover, the results from complementation analyses (Fig. 2) and from two-hybrid screens provide support that the product of the 2A5 gene from *Drosophila* is functionally equivalent to *S. cerevisiae* Atp11p.

Multiple alignment of the amino acid sequences shows that there is a significant level of sequence similarity in homologous Atp11p proteins across evolutionary lines (Fig. 1). The low percent identity (14.6%) among all four aligned sequences is consistent with our view that the molecular mechanism of Atp11p may be dependent more on overall conformation of the protein than on specific amino acid residues. Previous work showed that respiratory defective *atp11* mutants of *S. cerevisiae*, which were isolated following chemical mutagenesis of yeast, were found to have only nonsense mutations in the coding sequence for mature Atp11p [19]. To explain these observations we argued that amino acid replacements, which may have been sustained during mutagenesis, did not prevent Atp11p from assembling enough F_1 to support growth on the selective media used in the genetic screen. Thus, the low level of identity amongst homologous Atp11p proteins might reflect the fact that there are very few amino acids that are essential for the correct interaction of Atp11p with the F_1 β subunit.

Our model for Atp11p action suggests that the chaperone binds to unassembled F_1 β subunits and prevents aggregation of the protein. All Atp11p proteins are predicted to have a similar structure and mechanism. Furthermore, the sequence of the binding site on the β subunit for the chaperone is predicted to be similar in all species owing to the high degree of amino acid sequence conservation observed amongst F_1 β

Table 1
Strains and plasmids

Yeast strains/plasmids	Genotype/description	Source
Strains		
W303-1A	*Mat**a** ade2-1 his3-11,15 leu2-,112 ura3-1 trp1-1*	R. Rothstein, Columbia University, NY
W303ΔATP11	*Mat**a** ade2-1 his3-11,15 leu2-,112 ura3-1 trp1-1 atp11::HIS3*	[5]
Y190	*MAT**a** ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4Δ gal80Δ cyh'2 LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3 URA3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	Clontech
Plasmids		
pCUP2A5/CEN316	*D. yakuba* cDNA 2A5 cloned downstream the yeast *CUP1* promoter in the single copy vector pRS316	This study
pCUP2A5/YEp	*D. yakuba* cDNA 2A5 cloned downstream the yeast *CUP1* promoter in the multicopy vector YEp352	This study
pAS2-1/2A5(33–278)	Mature coding sequence for *D. yakuba* Atp11p fused with the Gal4p DNA binding domain sequence in pAS2-1	This study
pACT2/ATP2(36–511)	Mature coding sequence for F_1 β subunit fused with the Gal4p activation domain sequence in pACT2	This study

Table 2
Pairwise sequence analysis of homologous Atp11p proteins

	S. cerevisiae	*S. pombe*	*D. yakuba*	*D. melanogaster*
	Percent similarity			
S. cerevisiae	–	73.8	72.3	69.0
S. pombe	35.7	–	66.5	67.2
D. yakuba	28.4	29.1	–	99.6
D. melanogaster	29.6	24.1	95.3	–
	Percent identity			

The top right area denotes percent similarity overall between sequence pairs (identical and physico-chemically similar amino acids included). The bottom left area shows percent identity between sequence pairs.

Table 3
Mitochondrial ATPase activity in yeast that produce Atp11p from *D. yakuba*

Strain	ATPase activity (U/mg)		
	Minus oligomycin	Plus oligomycin	Oligomycin-sensitive activity
W303ΔATP11/CUP2A5/CEN316	0.36 (0.40)	0.21 (0.19)	0.18
W303ΔATP11/pCUP2A5/YEp	0.66 (0.83)	0.39 (0.20)	0.45

Mitochondria were isolated from yeast grown in YPGal to early stationary phase and ATPase activity was measured ± oligomycin as described in [5]. Results from two separate mitochondrial preparations are shown; one set of values obtained ± oligomycin is given in parentheses. The mean values for oligomycin-sensitive ATPase activity are shown.

subunits. For example, pairwise analysis between F_1 β subunits from *S. cerevisiae* (GenBank accession number Z49621) and from *D. melanogaster* (GenBank accession number X71013) indicates 90% similarity. In consideration of these points, one might predict that the *Drosophila* protein would be an excellent substitute for *S. cerevisiae* Atp11p in a yeast cell. However, the results from our complementation analyses indicate that the *Drosophila* Atp11p confers suboptimal levels of respiratory competence to a yeast *atp11* mutant. To explain this result we suggest that the sequence diversity observed amongst Atp11p homologs might also reflect evolution of the chaperone to meet the specific requirements of the host cell. For example, it is not known at this time if the action of Atp11p is regulated in mitochondria, or if the chaperone acts in conjunction with other proteins that have not yet been identified

Acknowledgements: This work was supported by National Institutes of Health Grant GM48157. We thank Domenico Gatti, Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, for critical evaluation of the manuscript.

References

[1] Penefsky, H.S. and Cross, R.L. (1991) Adv. Enzymol. 64, 173–214.
[2] Boyer, P.D. (1993) Biochim. Biophys. Acta 1140, 215–250.
[3] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Nature 370, 621–628.
[4] Bianchet, M.A., Hulihen, J., Pedersen, P.L. and Amzel, L.M. (1998) Proc. Natl. Acad. Sci. USA 95, 11065–11070.
[5] Ackerman, S.H., Martin, J. and Tzagoloff, A. (1992) J. Biol. Chem. 267, 7386–7394.
[6] Bowman, S., Ackerman, S.H. and Tzagoloff, A. (1991) J. Biol. Chem. 266, 7517–7523.
[7] Ackerman, S.H. and Tzagoloff, A. (1990) Proc. Natl. Acad. Sci. USA 87, 4986–4990.
[8] Roth, A.F. and Davis, N.G. (1996) J. Cell Biol. 134, 661–664.
[9] Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.
[10] Hill, J.E., Myers, A.M., Koerner, T.J. and Tzagoloff, A. (1986) Yeast 2, 163–167.
[11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[12] Fields, S. and Song, O.-K. (1989) Nature 340, 245–246.
[13] Schiestl, R.H. and Gietz, R.D. (1989) Curr. Genet. 16, 339–346.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[16] Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[17] Schmid, K.J. and Tautz, D. (1997) Proc. Natl. Acad. Sci. USA 94, 9746–9750.
[18] White, M. and Ackerman, S.H. (1995) Arch. Biochem. Biophys. 319, 299–304.
[19] Wang, Z.-G. and Ackerman, S.H. (1996) J. Biol. Chem. 271, 4887–4894.
[20] Hartl, F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochim. Biophys. Acta 988, 1–45.