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Identification of a 66 KDa protein associated with yeast mitochondrial ATP synthase as heat shock protein hsp60

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A 66 kDa protein, denoted P66, not hitherto classified as an integral component of yeast mitochondrial ATPase, is often observed in preparations of this enzyme complex. A physical association exists between P66 and the assembled ATPase complex since both components are coimmunoprecipitated by anti-$F_1\beta$ monoclonal antibody. Two recombinant clones expressing proteins immunologically similar to P66 were isolated from a yeast genomic library in λgt11 by screening with a polyclonal anti-holo-ATPase antibody. Based on restriction site mapping and partial nucleotide sequence analysis, both clones encompass the gene encoding the yeast heat shock protein hsp60. The identification of P66 with hsp60, taken together with its demonstrated association with the mitochondrial ATPase complex, is consistent with recent suggestions that hsp60 is involved in assembly of the ATP synthase complex.

Mitochondrial ATPase complex; Mitochondrial biogenesis; *HSP60*; *Saccharomyces cerevisiae*

1. INTRODUCTION

The mitochondrial ATP synthase complex (mtATPase) of the yeast *Saccharomyces cerevisiae* is a multisubunit enzyme complex composed of two distinct sectors [1]. The hydrophilic F_1 sector is required for nucleotide binding and the interconversion of ATP and ADP. The five subunits of this sector (α, β, γ, δ, ϵ) are encoded by the nucleus, synthesized in the cytoplasm as precursor proteins and subsequently imported into the organelle to be assembled into a functional complex. In contrast, the hydrophobic F_O sector is embedded within the inner mitochondrial membrane to form a proton channel and contains three mitochondrially encoded subunits (6, 8, 9). A third group of mtATPase-associated proteins, collectively denoted F_A [1], is comprised of proteins thought to make structural and functional links between the F_1 and F_O sectors. Members of this class include an oligomycin sensitivity conferring protein, OSCP [2], and two further mtATPase-associated proteins, P18 and P25 [3].

Additionally, preparations of mtATPase often include a 66 kDa protein which has been designated P66. In this study, we show that P66 is physically associated with the mtATPase. In addition, molecular cloning and characterization of yeast DNA encoding P66 reveals its identity with the yeast heat shock gene, *HSP60*.

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2. MATERIALS AND METHODS

Mitochondrial ATPase was prepared from purified mitochondria either by separation of Triton X-100 extracts on 5-15% glycerol gradients [5] or by separation of cholate/octyl β-D-glucopyranoside extracts on 7-30% sucrose gradients [6]. Radiolabelled mtATPase was isolated from cells incubated in the presence of [^{35}S]methionine through the use of an anti-$F_1\beta$ monoclonal antibody capable of immunoadsorbing the entire ATPase complex from Triton X-100 extracts of purified mitochondria [3]. The ATPase activity was determined using a coupled assay [3]. Affinity-purification of specific antibodies and the use of this methodology to identify genes on cloned DNA fragments has been described [7]. Proteins were electrophoretically separated by SDS-PAGE on 12.5% gels and visualized by Coomassie staining or fluorography [3]. Western immunoblots were performed as described [8] using Tween-20 as a blocking agent. All DNA manipulations were accomplished using established techniques [9]. The nucleotide sequence of a λgtll insert recloned into pUC19 was determined using the double-stranded plasmid as template [10]. The two synthetic oligonucleotide primers employed in the sequence analysis were homologous to the reading frame of the yeast *HSP60* gene [4]; one primer was identical to nucleotides -4 to +16 while the second primer was complementary to nucleotides +1705 to +1726 of the published sequence.

3. RESULTS AND DISCUSSION

On a typical glycerol gradient used during the purification of mtATPase from yeast mitochondrial lysates, the ATPase activity is localized to fractions 5-7, with fraction 6 containing greater than 50% of the total activity (Fig. 1A). Electrophoretic analysis indicates that fraction 6 is enriched for a small number of prominent polypeptides comprising the F_1, F_O, and F_A sectors of the mtATPase (Fig. 1B). In general, the

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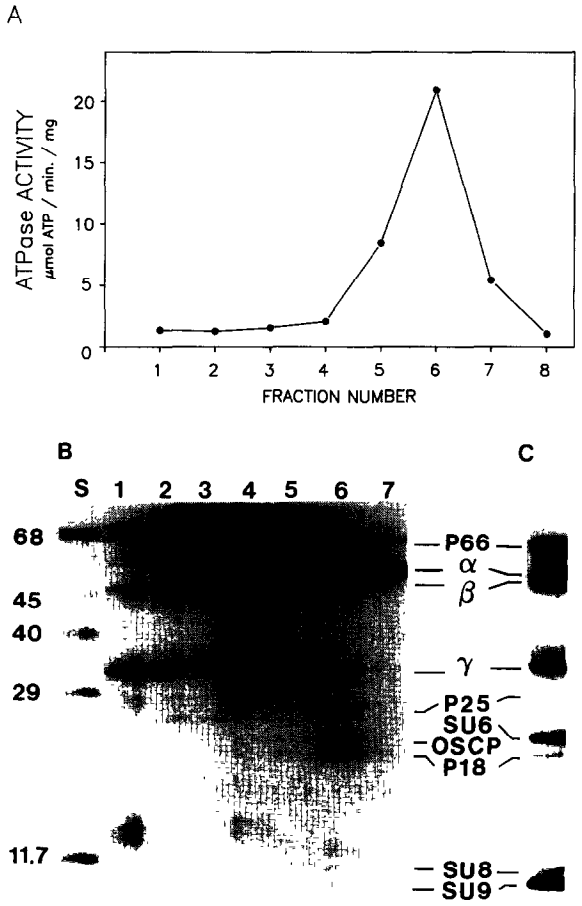

Fig. 1. Composition of the yeast mtATPase. Yeast MtATPase was purified by glycerol gradient separation of a mitochondrial lysate. After fractionation into ten fractions of equal volume, the ATPase activity was localized (A) and 200 mg of protein from each fraction was analyzed by SDS-PAGE (B). Lane numbers refer to fraction numbers, starting with fraction 1 at top of gradient. The protein standards (lane S) are bovine serum albumin (66 kDa), ovalbumin (45 kDa), aldolase (40 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (11.7 kDa). Radiolabeled mtATPase was isolated from mitochondrial lysates by immunoabsorption to an anti-F_1 β antibody coupled to Sepharose beads [3] prior to SDS-PAGE and fluorography (C). Some of the mtATPase subunits are indicated.

quantity of these polypeptides correlates well with the ATPase activity associated with each fraction.

Another polypeptide found to be enriched in fraction 6 has an apparent molecular mass of 66 kDa and has been designated P66. Although P66 is present in most glycerol gradient-purified preparations of mtATPase, the yield of this protein relative to that of authenticated mtATPase subunits is variable. In general, P66 behaves similarly during both glycerol and sucrose gradient-based schemes for mtATPase purification. Thus, P66 clearly co-fractionates with the mtATPase during sedimentation gradient centrifugation, although its relationship to the complex is unclear.

Evidence that P66 is physically associated with the mtATPase has been obtained using an assay [3] which utilizes a monoclonal antibody specifically recognizing the β subunit of holo-mtATPase. This anti-$F_1\beta$ antibody specifically immunoadsorbs the entire mtATPase complex from detergent lysates of purified mitochondria [1,3]. Electrophoretic separation of the immunoadsorbed proteins (in this case from cells radiolabelled with [^{35}S]-methionine) results in the visualisation (Fig. 1C) of the subunits comprising the F_1, F_O and F_A sectors of the mtATPase complex. In addition, a prevalent P66 band is also evident. Thus, by virtue of the co-immunoadsorption of P66 with the mtATPase, we conclude that a physical link exists between P66 and the complex. A P66 band is also clearly visible amongst the proteins in unlabelled immunoprecipitates, stained with Coomassie blue or silver (data not shown).

As a first step in examining the nature and function of P66, DNA clones encoding P66 were isolated from a yeast genomic library constructed in the λ-phage expression vector, λgtll. In a previous study [11], this library was screened with a polyclonal anti-holo-mtATPase antiserum, designated M56, and 54 immunoreactive clones were isolated. It was anticipated that the gene encoding P66 would be represented among these clones, since P66 is detected when Western blots of electrophoretically separated mtATPase are probed with the M56 antiserum (Fig. 2, lane 2).

Pools of λgtll clones, carrying related DNA inserts identified by dot blot hybridization analysis, were established [11] and then employed to immunopurify specific antibodies from the M56 antiserum. Upon probing Western blots of mtATPase with the immunopurified antibodies, a specific relationship could be established between the product of a cloned DNA fragment and a mtATPase subunit or associated protein. Using this approach, the group comprising clones 17 and 34 was identified as producing a protein antigenically similar to P66 (Fig. 2, lane 3). Note that the identification of the 54 kDa protein by antibodies immunopurified using clones 17 and 34 was not insert-dependent as this occurred even when λgtll without a yeast insert was used (Fig. 2, lane 4). The identity of this material is not understood [11].

The inserts from clones 17 and 34 were subcloned into pVC 19 as 3.7 and 4.0 kb *Eco*RI fragments, respectively. Subsequent restriction site mapping indicated that the clone 17 insert is a sub-fragment of the clone 34 insert; a restriction map for the clone 34 insert is shown (Fig. 3). One additional recognition site for each of *BclI* and *PstI* was mapped at the genomic location of the P66-related DNA by probing Southern blots of total yeast DNA digested with *BclI* or *PstI* with clone 34 insert DNA labelled by nick-translation. Similar analysis indicates that the P66-related DNA resides on a 5.2 kb genomic *Eco*RI fragment (data not shown).

Significantly, the restriction site map of the P66-related DNA closely resembles that of the recently

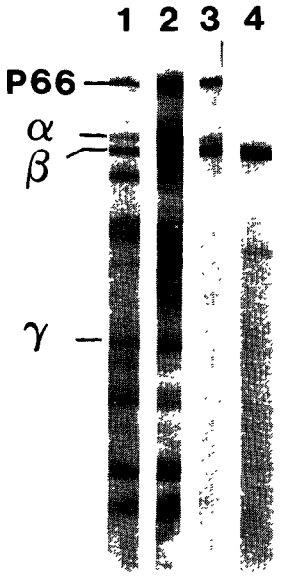

Fig. 2. Identification of clones encoding P66-related protein. Purified mtATPase [6] was Western blotted and probed with a polyclonal antibody raised against holo-mtATPase (lane 2), antibodies immunopurified using clones 17 and 34 (lane 3) or, in an independent experiment, antibodies immunopurified using λgt11-infected *E. coli* (lane 4). The Indian ink-stained profile of purified mtATPase is shown for comparison (lane 1).

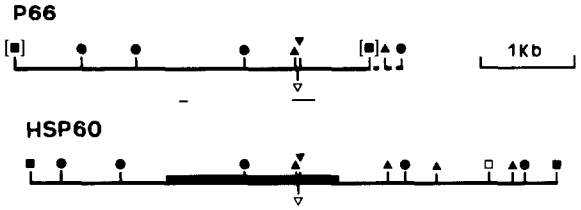

Fig. 3. Restriction maps of DNA segments encoding P66 and hsp60. Schematic for P66 is based on the gel mobilities of restriction fragments (thin horizontal line, map of λgt11 insert from clone 34; dashed line, flanking chromosomal DNA mapped by genomic Southern blot). Lines under the map designate extent of sequence analysis. Sites in the *HSP60* gene map are based on the reported nucleotide sequence [4]. Key to sites: [■], *Eco*RI originating from synthetic linkers; ■, *Eco*RI; •, *BclI;* ▲, *Pst*I; ▽, *Xba*I; ▼, *Sal*I; □, *Bam*HI. The thick black bar represents the coding sequence for the *hsp60* gene (transcribed from left to right).

described *HSP60* heat shock gene of yeast which is located on a 5.3 kb genomic *Eco*RI fragment (Fig. 3). To confirm that the P66-related DNA carries the *HSP60* sequence, synthetic oligonucleotides complementary to the 3' and 5' ends of the *HSP60* reading frame were used as primers to sequence in part the P66-related DNA (Table I). The resulting nucleotide sequences were found to be identical to the corresponding sequences from the 5' and 3' ends of the *HSP60* reading frame, respectively, (Table I), strongly suggesting that the 4.0 kb fragment of yeast DNA inserted into λgtll clone 34 represents the *HSP60* gene. This proposition was confirmed by the finding that P66-related DNA, when incorporated into low- or multi-copy vectors, is able to complement at the restrictive temperature the growth defect of a strain carrying a temperature conditional *mif4* mutation [13] in the *HSP60* gene (data not shown). The presence of either a *mif4* mutation at the restrictive temperature or a desrupted *HSP60* gene confers a lethal phenotype on haploid yeast strains [13]. Thus, it may be reasonably concluded that the yeast genome harbors only a single gene encoding hsp60 function.

We have isolated this gene from a yeast genomic library by virtue of the immunological relatedness between a mtATPase-associated protein, P66, and the putative product expressed in bacteria by a 4.0 kb genomic DNA fragment evidently encompassing the *HSP60* locus. Three additional observations support the conclusion that *HSP60* encodes P66. Firstly, from SDS-PAGE analysis, the estimated molecular weights of hsp60 and P66 are similar, being 64 [4] and 66 kDa, respectively. Note that the M_r of hsp60 based on its predicted sequence [4], including a putative N-terminal signal peptide for mitochondrial import, is about 61,000. Secondly, both P66 and hsp60 are mitochondrial proteins, with hsp60 being associated with the matrix compartment of the organelle [12,13]. Finally the physical association of P66 with the mtATPase is consistent with the proposed involvement of the hsp60 protein in the assembly of multi-subunit mitochondrial protein complexes [13].

The representation of the P66 band in purified mtATPase preparations is variable (data not shown here). Although its abundance in the complex may be a function of the particular purification procedure used and the genetic or physiological status of the cells, it has not yet been possible to identify the source of its variable representation. The inconsistent presence and yield of P66/hsp60 in mtATPase preparations can now be viewed in the context of the probable role of this protein in the assembly of the mtATPase [13]. For example, yeast cells requiring higher levels of newly assembled mtATPase may be expected to contain a greater proportion of mtATPase associated with hsp60 than cells lacking this requirement. Therefore, the variable presence and yield of hsp60 in mtATPase preparations may be due to variations in the mtATPase requirements

Table I
Confirmation of the P66-related DNA as *HSP60* by partial nucleotide sequencing

Region	Primer sequence[a]	Sequence verified as *HSP60*[a]
5'; 'Upstream'	nucleotides -4 - 16	nucleotides 124 - 216
3'; 'Downstream'	nucleotides 1705 - 1726	nucleotides 1300 - 1530

[a]Nucleotide numbers correspond to those of the *HSP60* nucleotide sequence reported by Reading et al. [4].

of the cells from which the enzyme complex has been prepared. Exploration of this possibility will be the subject of future investigations.

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