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Identification of Mitochondrial and Microsomal Phosphatidylserine Synthase in *Saccharomyces cerevisiae* as the Gene Product of the *CHO1* Structural Gene

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In *Saccharomyces cerevisiae*, the membrane-associated enzyme phosphatidylserine synthase (EC 2.7.8.8) is present in the mitochondria and the endoplasmic reticulum. The enzyme from both membrane fractions reacted with antiserum raised against a hybrid protein expressed from a *TRPE-CHO1* fusion gene in *Escherichia coli* and was absent in a *cho1* null mutant, strongly suggesting that both the mitochondrial and microsomal forms of phosphatidylserine synthase are the products of the *CHO1* gene. The highest degree of purification of enzymatically active protein was 380- and 420-fold from the mitochondrial and the microsomal compartments, respectively. In both cases, the enzymatically active and immunoreactive material comigrated with a protein band of 30,000 apparent molecular weight. In the absence of protease inhibitors during the preparation of membranes, the enzyme underwent degradation to an enzymatically active protein of 23,000 apparent molecular weight.

Phosphatidylserine synthase (PSS [CDPdiacylglycerol–serine O-phosphatidyltransferase, EC 2.7.8.8]) is an integral membrane protein. In *Saccharomyces cerevisiae*, its product, phosphatidylserine, is involved in the synthesis of the major cellular phospholipids phosphatidylethanolamine and phosphatidylcholine. Detailed biochemical studies have demonstrated that PSS activity is present both in the mitochondria and in the endoplasmic reticulum (5). *cho1* mutants lacking total cellular PSS activity have been selected as choline auxotrophs (1). Analysis of these mutants and the construction of null mutants of the *CHO1* gene established that only one chromosomal copy of the gene exists and excluded the presence of isoenzymes of PSS. Therefore, it appeared likely that mitochondrial and microsomal PSS are encoded by the same structural gene (3; S. D. Kohlwein, K. Kuchler, A. M. Bailis, F. Paltauf, and S. A. Henry, submitted for publication). Although biochemical, molecular, and genetic studies strongly suggested that *CHO1* is the structural gene for PSS (3, 6; Kohlwein et al., submitted for publication), a direct demonstration that *CHO1* encodes phosphatidylserine synthase has not been presented. The existence of but a single gene also raises an important question. That is, by what mechanism is the product(s) of one gene directed to two subcellular membrane compartments?

PSS was originally purified to homogeneity from microsomes of glucose-grown yeast cells, and the purified enzyme had an apparent molecular weight of 23,000 (2). In order to characterize the mitochondrial and microsomal forms of PSS, we attempted to purify the enzyme from the mitochondria and microsomes separately. Using published methods (2, 5), we achieved 380- and 420-fold purifications of enzymatically active protein from mitochondria and microsomes, respectively (Table 1). Further purification steps, including ion-exchange chromatography on Whatman DE-53 resin, resulted in a rapid loss of enzymatic activity. To correlate enzyme activity with the molecular weight, we employed the electroblotting procedure described by Poole et al. (7). After separation of membrane proteins on denaturing polyacrylamide gels (12.5%) and transfer to nitrocellulose filters, PSS was renatured in the presence of CDP-diacylglycerol. The filters were cut into 5-mm slices, and enzyme activity was assayed in each slice. PSS activity distributions in mitochondrial and microsomal preparations of wild-type strain DCZ8-7C (*MAT*α *leu1*) are shown in Fig. 1. To our surprise, PSS activity was associated not only with a protein of 23,000 molecular weight, as reported in the literature (2, 8), but also—and for the most part—with a 30,000-molecular-weight protein. The ratio of the 30,000- and 23,000-molecular-weight forms varied between fractions, but the low recovery of activity after blotting did not allow us to make exact quantitative comparisons. It appeared unlikely that the two different sizes of active PSS were the result of proteolytic processing during the course of intracellular targeting, since both forms of the enzyme were present in both compartments. Cross contamination between mitochondrial and microsomal fractions could also be excluded, since analysis of marker enzymes demonstrated that contamination was below 5%.

Among the various explanations for the existence of two forms of PSS which can be considered are the following. (i) *CHO1* might be a regulatory gene rather than a structural gene, affecting the expression of two different genes coding for mitochondrial and microsomal PSS, respectively. (ii) PSS might be translated from different transcripts of the same structural gene, as it was reported for the intracellular and secreted forms of invertase, the gene products of the *SUC2* gene (4). (iii) Translation might start at different sites of a uniquely sized *CHO1* transcript. (iv) The occurrence of a shorter polypeptide might be the consequence of proteolytic degradation during the preparation procedure.

To elucidate the nature of the primary translation product

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TABLE 1. Purification of a 30-kilodalton phosphatidylserine synthase from mitochondria and microsomes of *Saccharomyces cerevisiae* DCZ8-7C (*Mat*α *leu1*)

Protein source	Protein (mg)	Specific activity[a]	Yield (%)	Enrichment[b]
Mitochondria				
Total homogenate	272	2.0	100	1
Triton X-100 extract	157	3.4	91	1.4
CDP-DAG Sepharose	0.46	841	71	421
DE-53 ion exchange	0.35	780	50	390
Microsomes				
Total homogenate	390	1.5	100	1
Triton X-100 extract	252	2.1	91	1.4
CDP-DAG Sepharose	0.61	575	62	383
DE-53 ion exchange	0.42	543	39	362

[a] Nanomoles of phosphatidylserine produced per minute and milligram of protein.
[b] Factor was set to 1 for total homogenate.

of the *CHO1* gene and its putative role in expression of mitochondrial and microsomal PSS, we constructed a *TRPE-CHO1* fusion gene in a pATH11 expression vector (kindly donated by T. J. Koerner) as summarized in Fig. 2a. The 0.9-kilobase *Eco*RI-*Pvu*II fragment of the *CHO1* gene, which was isolated by its ability to restore choline prototrophy to a *cho1* mutant (6), was inserted into the *Eco*RI-*Sma*I site of the multiple cloning site of the expression vector. This resulted in an in-frame fusion of *CHO1* (Kohlwein et al., submitted for publication). The hybrid gene was expressed in *Escherichia coli* in the presence of indoleacrylic acid. Total cellular proteins and insoluble fraction were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gels. After transfer to nitrocellulose filters, immunoreactive hybrid protein was visualized by use of antiserum (kindly provided by G. Carman) raised against the purified 23,000 subunit of PSS and by incubation with ^{125}I-protein A (Fig. 2b). In the transformants, a hybrid protein of 67,000 molecular weight was identified after induction (Fig. 2b, lanes E). The corresponding band on the autoradiograph of the immunoblot (Fig. 2b, right panel, lane E) demonstrated the identity of epitopic areas on the 23,000-molecular-weight subunit of PSS and the hybrid protein expressed in *E. coli*. This immunoreactive protein was absent in uninduced cells harboring the *TRPE-CHO1* fusion gene (Fig. 2b, right panel, lane D) or in wild-type *E. coli* (Fig. 2b, right panel, lanes A and B). This observation provides unequivocal proof that *CHO1* represents the structural gene of yeast PSS. The size of about 67,000 for the hybrid protein suggested a calculated molecular weight of about 30,000 for the PSS portion. There is, however, cross-reactive material corresponding to a molecular weight of about 60,000 which might be a proteolytic degradation product. Immunoreactive materials of about 35,000, 58,000 and 94,000 molecular weights, respectively, are present in all preparations and represent cross-reactive material from *E. coli*. Both bands at 67,000 and 60,000 apparent molecular weights are present only in induced *E. coli* cells harboring the fusion gene. Hybrid protein of 67,000 molecular weight was purified from induced *E. coli* by standard procedures and injected into Chinchilla rabbits to raise antibodies for further biochemical studies.

Immunoblots of isolated mitochondrial and microsomal membranes (5) with antibody raised against TRPE-PSS hybrid protein are presented in Fig. 1. Both cellular fractions exhibit most of the immunoreactive material associated with the 30,000-molecular-weight protein, which is a confirmation of the activity distribution of the enzyme as obtained by the electroblotting experiments (Fig. 1). Total membranes from *cho1* null mutants lack any detectable immunoreactive material in the range of 30,000 and 23,000 apparent molecular weights, which confirms that both subcellular forms of PSS are encoded by the same structural gene (Fig. 1, lane 5).

Total membranes of yeast cells prepared by a rapid-disruption method with glass beads in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (5) lacked any immunoreactive PSS of 23,000 molecular weight as determined by using antiserum against TRPE-PSS hybrid protein (Fig. 3). The appearance of an immunoreactive fraction of about 56,000 molecular weight, which most likely represents CDP-diacylglycerol synthase (G. Carman, per-

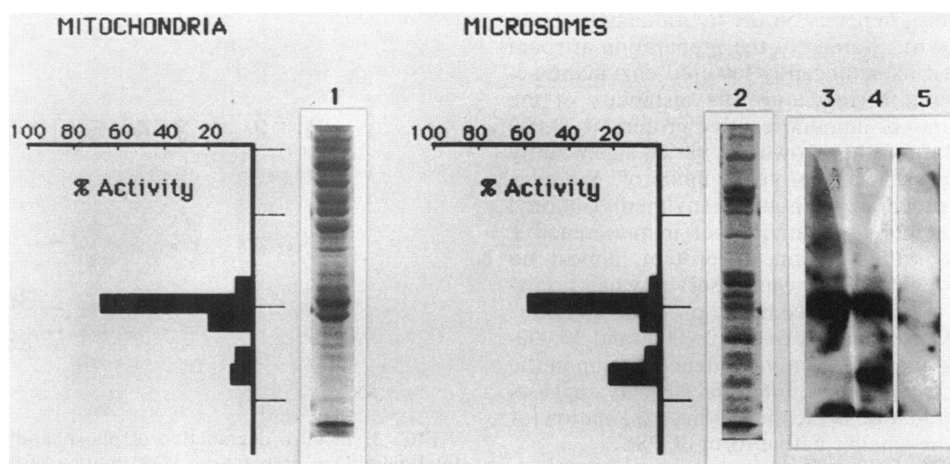

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining of isolated mitochondrial and microsomal membrane preparations from wild-type strain DCZ8-7C (*MAT*α *leu1*), transfer of proteins to nitrocellulose membranes and activity distribution (graphs to left of lanes 1 and 2) of phosphatidylserine synthase in mitochondria (lane 1) and microsomes (lane 2) after electroblotting to nitrocellulose. Horizontal lines mark the sizes of molecular weight standards (from top to bottom: 67,000, 43,000, 30,000, and 20,100). Immunoblots of the respective membranes are shown in lane 3 (mitochondria) and lane 4 (microsomes). Lane 5 shows an immunoblot of total membranes prepared from a *cho1* null mutant (3). Immunohybridizations were performed with antiserum raised against TRPE-PSS hybrid protein expressed and purified from *E. coli*.

FIG. 2. Construction of a *TRPE-CHO1* fusion gene and expression in *E. coli*. (a) Construction of a *TRPE-CHO1* fusion-gene. The *Eco*RI-*Pvu*II fragment of *CHO1* (pAB306 [3]) was inserted into the multiple cloning site of pATH11. The in-frame fusion leads to a hybrid protein of 67,000 apparent molecular weight (bar). bp, Base pair; ORF, open reading frame; kd, kilodalton. (b) Detection of the TRPE-PSS hybrid protein in induced *E. coli* harboring the *TRPE-CHO1* fusion gene by Coomassie blue staining (left panel) and immunoblotting (right panel) with antiserum against purified phosphatidylserine synthase of 23,000 molecular weight (kindly provided by G. Carman). Lanes: St, molecular weight standards (from top to bottom: 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400); A, total extract of *E. coli* HB101; B, total extract of induced HB101; C, total extract of induced HB101 harboring the *TRPE* gene on an episomal plasmid; D, insoluble fraction of HB101 harboring the *TRPE-CHO1* fusion gene; E, insoluble fraction of the induced HB101 harboring the *TRPE-CHO1* fusion gene.

sonal communication), depends on the hybridization conditions (data not shown). Storage of the preparation at room temperature resulted in significantly lowered enzymatic activity of the preparation, indicating the instability of the enzyme. The amounts of immunoreactive protein at 23,000 and 30,000 molecular weights, however, varied significantly depending on the presence of protease inhibitor. Whereas total membranes prepared without phenylmethylsulfonyl fluoride showed an increased amount of immunoreactive material associated with the smaller protein, almost no 23,000-molecular-weight protein was observed when preparations were made in the presence of the protease inhibitor. This modulation of the amounts of the 23,000- and 30,000-molecular-weight forms of PSS in vitro depending upon the presence or absence of protease inhibitor strongly suggests that proteolytic degradation is the underlying explanation for the occurrence of the smaller active form of PSS.

Detailed studies, including S1 nuclease mapping of transcription starts (3; Kohlwein et al., submitted for publication), show that in wild-type cells, a uniquely sized transcript of about 1.2 kilobases is expressed, differing only in a few bases at the 5′ end. Thus, different transcription initiation sites are not involved in selective targeting and regulatory mechanisms for expression of mitochondrial and microsomal

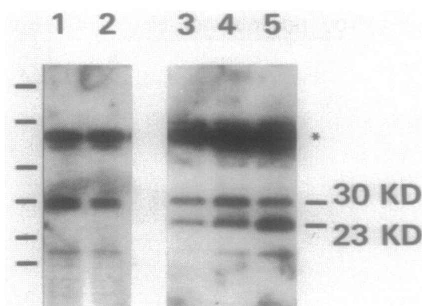

FIG. 3. In vitro degradation of phosphatidylserine synthase in total membrane preparations. Distribution of immunoreactive protein in total membranes with (lanes 1 and 2) or without (lanes 3 to 5) 1 mM phenylmethylsulfonyl fluoride and after 2 h (lane 4) and 6 h at room temperature (lanes 2 and 5). Cross-reactive material of about 56,000 apparent molecular weight (*) most likely represents CDP-diacylglycerol synthase (G. Carman, personal communication). Horizontal lines indicate molecular weight standards (from top to bottom: 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400).

forms of PSS and cannot explain the existence of the 23,000- and 30,000-molecular-weight forms.

The final possibility that different sizes of PSS in vivo may result from more than one translation initiation site cannot be ruled out with certainty, since sequencing and detailed experiments revealed a second potential translation start which is active both in vitro and, under certain circumstances, in vivo (Kohlwein et al., submitted for publication). However, in wild-type cells, the second putative translation initiation site seems to be very ineffective. Attempts are under way to prepare mitochondrial and microsomal phosphatidylserine synthase in pure form to allow amino acid sequencing of the N terminus. This should provide definitive proof that the mitochondrial and microsomal forms of PSS are identical at their N termini.

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ADDENDUM IN PROOF

In a recent paper, Kiyono et al. (K. Kiyono, K. Miura, Y. Kushima, T. Hikiji, M. Fukushima, I. Shibuya, and A. Ohta, J. Biochem. **102**:1089–1100, 1987) reported on the overexpression of *CHO1* and characterization of the gene product. They found that a 30-kilodalton protein is the primary translation product which can be proteolytically degraded to an enzymatically active 23-kilodalton protein. They speculate that the 30-kilodalton protein is not a functional PSS, but was its precursor.

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