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A yeast mutant lacking mitochondrial porin is respiratory-deficient, but can recover respiration with simultaneous accumulation of an 86-kd extramitochondrial protein

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A yeast mutant lacking the only known pore-forming protein of the mitochondrial outer membrane was constructed by gene disruption. The mutant retained all other major proteins of the mitochondrial outer membrane, but was severely deficient in mitochondrial cytochromes and initially did not grow on the non-fermentable carbon source, glycerol. However, it could slowly adapt to glycerol; adaptation was accompanied by the partial restoration of cytochrome levels and massive accumulation of an 86-kd polypeptide in extramitochondrial cell fractions.

Key words: outer membrane pore/yeast mitochondria/gene disruption/respiration defect

Introduction

Formation and function of mitochondria require complex interactions between these organelles and the surrounding cytosol. Many of these interactions are mediated by mitochondrial proteins which allow the passage of specific molecules (or groups of molecules) across the mitochondrial membranes. For example, transport of charged metabolites across the inner membrane is catalyzed by specific carriers such as the phosphate translocator or the adenine nucleotide translocator (Klingenberg, 1980). Transport of cytoplasmically made proteins into mitochondria most likely requires proteinaceous surface receptors and, presumably, a translocating machinery in the inner membrane (Teintze and Neupert, 1984; Hay *et al.*, 1984). Finally, transport of most (if not all) metabolites across the outer membrane occurs through a major pore. Although this pore exhibits some anion selectivity and voltage dependence (Benz, 1985), it appears to allow passage of all molecules whose mol. wt is <6000; it may, thus, be the major gate through which metabolites move between mitochondria and the cytoplasm.

More detailed characterization of these translocators and the outer membrane pore is essential if we wish to understand how mitochondria are made and how they function. To this end, we have constructed a yeast mutant lacking the outer membrane pore.

In yeast, the mitochondrial outer membrane pore is composed of a single type of subunit (termed 'porin') which is the most abundant protein of the mitochondrial outer membrane (Riezman *et al.*, 1983). It is coded by a nuclear gene and imported into the outer membrane without proteolytic cleavage (Hay *et al.*, 1984). The apparent mol. wt of porin is 29 kd as determined by SDS–PAGE. The porin gene has been cloned (Suissa *et al.*, 1984; Mihara and Sato, 1985) and sequenced (Mihara and Sato, 1985); the predicted amino acid sequence of yeast porin corresponds to a mol. wt of 29 883, in good agreement with the electrophoretically determined value.

In this study we have used the cloned porin gene to selectively disrupt the porin gene in the yeast nucleus. The resulting porin-free yeast mutant is viable, but respiration-deficient. Closer study of its unexpected properties should yield new information on the role of the outer membrane pore in mitochondrial function and mitochondrial biogenesis.

Results

Disruption of the porin gene in the yeast nucleus

We had earlier cloned the yeast porin gene based on its hybridiz-

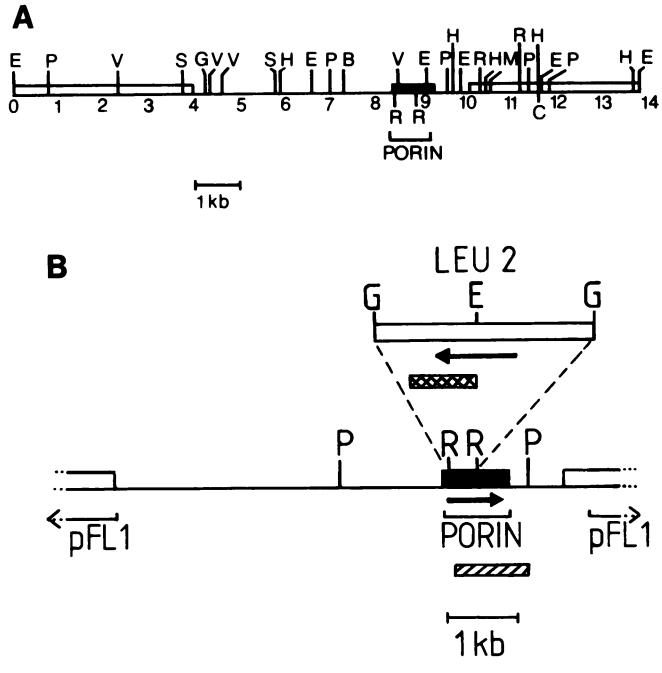

Fig. 1. The cloned nuclear yeast gene encoding mitochondrial porin. **(A)** Restriction maps of the cloned fragment of yeast genomic DNA in the vector pFL1. Open bar, DNA of plasmid pFL1 and (in part **B**) of the *LEU2* gene; single line, cloned fragment of genomic yeast DNA; filled bar, porin gene. B = *Bam*HI; C = *Cla*I; E = *Eco*RI; H = *Hin*dIII; M = *Sma*I; P = *Pst*I; R = *Eco*RV; S = *Sal*I; V = *Pvu*II. **(B)** Strategy for disrupting the porin gene in the yeast nucleus. A 3-kb *Bgl*II fragment containing the yeast *LEU2* gene was made blunt-ended with the Klenow fragment of DNA polymerase and inserted into the gap generated by excision of a 444-bp *Eco*RV fragment from within the porin gene. A 5-kb *Pst*I fragment carrying the disrupted porin gene was used for transformation of yeast cells. Hatched bar: porin DNA used as probe in the Southern blot hybridization; cross-hatched bar, *LEU2* DNA used as probe for Southern hybridization. Other symbols as in part **A**.

Table I. Isolation of por$^-$ mutants by gene disruption

	No. of colonies obtained from:	
	MAT α HR 125-5D	MAT *a*/MAT α HR 125-5D
Leu$^+$ in first screen	24	14
Leu$^+$ in second screen	6	4
por$^-$ found by immune or Southern blotting	1	3

Table II. Viability of the porin-deficient mutant does not depend on an unlinked suppressor mutation

	% of asci with the following number of viable spores					Total spore viability (%)	No. of tetrads dissected
	4	3	2	1	0		
Expected values if viability were caused by an unlinked suppressor	16.7	66.7	16.7	0	0	75	Not applicable
a HR 125 × αHR 125	25	28	39	5	3	66	36
a HR 125 × αHR 125 por^-	41	25	18	9	7	71	44
a/α HR 125 por^-	40	20	40	0	0	75	10
a HR 125 por^- × αSF 747	78	22	0	0	0	95	23

ation to porin mRNA. This clone apparently contains the entire porin gene: when introduced into yeast on a multi-copy plasmid it caused 2- to 3-fold overproduction of porin as detected by immunoblotting (Suissa *et al.*, 1984). The yeast porin gene was independently cloned by Mihara and Sato (1985), who also determined the nucleotide sequence of the gene. Based on this information, we disrupted the nuclear porin gene in a *leu2* auxotroph by deletion of the 5′ half of the gene and insertion of a functional *LEU2* gene. Both haploid and isogenic diploid cells were subjected to gene disruption. Figure 1 gives a restriction map of the porin clone isolated by Suissa *et al.* (1984) and our strategy for gene disruption. Only the first eight amino acids of porin (total length: 283 residues) should still be made in the resulting mutant.

We obtained 24 haploid leucine-independent transformants but only six of these retained their Leu^+ phenotype upon rescreening (Table I). Only one of those lacked porin as measured by immunoblotting of total cellular proteins (not shown). In addition, we obtained 14 diploid Leu^+ transformants, four of which were stable; three of these contained one copy of a disrupted porin gene (by Southern analysis; not shown).

Both diploid POR^+/por^- mutants and the haploid por^- mutant were viable. The haploid por^- mutant grew on glucose, but more slowly than the POR^+ parent, particularly at 37°C. It failed to grow on glycerol/ethanol for at least 2–3 days, but started to adapt to these non-fermentable carbon sources after 3 days (see below).

When the haploid por^- mutant was crossed to its isogenic POR^+ parent or to an unrelated POR^+ strain, analysis of the resulting spores showed strict cosegregation of the Leu^+ phenotype, lack of porin and initial inability to grown on glycerol/ethanol. Of 32 tetrads with four viable spores, all showed 2:2 cosegregation of Leu^-:Leu^+ and the other two traits (see also below). This suggests that all three effects are caused by the mutation of a single nuclear gene. We checked whether the por^- mutant carried an independent, unlinked suppressor gene that might render the mutant viable. No such suppressor was found. If such a suppressor were present, it should be separable from the por^- mutation by backcrossing; upon crossing the por^- mutant to a POR^+ strain, 25% of the spores issued from the cross should be non-viable. Initially this was difficult to test since crosses between the por^- mutant and the isogenic POR^+ parent (HR 125-5D) were plagued by low spore viability. This was apparently unrelated to the por^- mutation as the same low spore viability was found in crosses between the two isogenic POR^+ parents (Table II). However, excellent (95%) spore viability was found upon crossing the por^- mutant to the unrelated POR^+ strain SF 747-19D; in those crosses exactly half the spores lacked porin. The same result was obtained with spore tetrads from the original diploid transformants (Table II). It is thus very unlikely that the viability of the porin-deficient cells is caused by an adventitiously selected unlinked suppressor mutation.

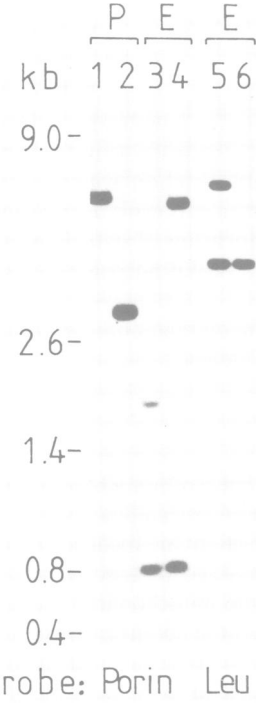

Fig. 2. The single nuclear porin gene is disrupted in the porin-deficient yeast mutant: genomic DNA from por^- **(lanes 1, 3** and **5)** and wild-type cells **(lanes 2, 4** and **6)** was digested with *Pst*I **(lanes 1** and **2)** or *Eco*RI **(lanes 3, 4, 5** and **6)**, separated on a 0.7% agarose gel and transferred to a 'Gene Screen Plus' membrane. Hybridization was carried out at 45°C in the presence of 50% formamide, 5 × SSC, 50 mM sodium phosphate, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 1% milk powder and 250 μg/ml of denatured salmon sperm DNA, with the ^{32}P-labeled DNA fragments outlined in Figure 1B as a probe. Note that the *LEU2* gene contains an *Eco*RI site and that the *LEU2* probe hybridizes to the left hand (5′) fragment, the porin probe to the right hand fragment in *Eco*RI digests.

When genomic DNA of the mutant and its parent strain were probed with labeled fragments of the porin gene or of the *LEU2* gene (Figure 1B) by 'Southern analysis', the hybridization pattern (Figure 2) conformed to that predicted by Figure 1B. Thus the mutant carries an inserted *LEU2* gene close to the start of its porin gene.

The por^- mutant has normal levels of other major outer membrane proteins, but is deficient in cytochromes

Total cellular proteins of the por^- mutant and the POR^+ parent strains were analysed for various mitochondrial proteins by immunoblotting. The results (Figure 3) were as follows: first, the por^- mutation completely eliminated immunologically detect-

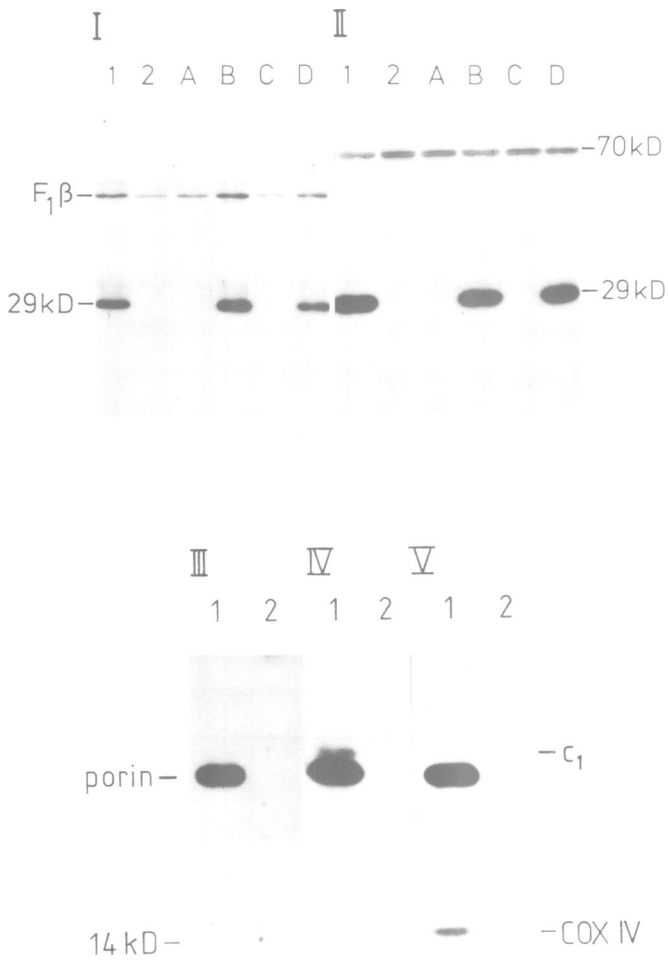

Fig. 3. The yeast mutant lacks mitochondrial porin. The POR^+ parent strain **(lanes 1)**, the por^- mutant **(lanes 2)** and four siblings from a cross between the two strains **(lanes A–D)** were grown on rich medium containing 2% glucose and the extracted cellular proteins (100 μg/gel lane) were analysed by SDS–12.5% polyacrylamide gel electrophoresis and immunoblotting for porin (29 kd) and the following additional mitochondrial proteins: **I**, β-subunit of F_1-ATPase ($F_1\beta$); **II**, 70-kd outer membrane protein (70 kd); **III**, 14-kd outer membrane protein (14 kd); **IV**, cytochrome c_1 (c_1); **V**, cytochrome *c* oxidase subunit IV (COX IV).

able porin from the cells, but did not alter the levels of several other major proteins of the mitochondrial outer membrane (shown for the 70- and 14-kd proteins but confirmed for three other outer membrane proteins, data not shown). Second, the level of the F_1-ATPase β-subunit was only reduced 1.6-fold. Third, porin-deficiency segregated 2:2 upon meiosis (lanes A–D in Figure 3, I,II). Fourth, the por^- mutation greatly lowered the levels of cytochrome c_1 and cytochrome oxidase subunit IV. Low-temperature difference spectra of por^- and POR^+ mitochondria confirmed that mitochondria from the por^- mutant grown on glucose contained greatly depressed levels of cytochromes *c*, c_1, *b* and aa_3 (Figure 4, left panels; note the higher sensitivity and higher mitochondrial concentration used for recording spectra of the por^- mitochondria). In agreement with the spectral data, specific cytochrome oxidase activity of mitochondria from glucose grown cells was ~10-fold lower in por^- mitochondria (0.091 μmol/min/mg mitochondrial protein) than in mitochondria from the isogenic POR^+ parent (0.97 μmol/min/mg).

While the lower cytochrome *c* levels could simply reflect loss of cytochrome *c* owing to increased fragility of the mutant mitochondria, such preferential loss is extremely unlikely for the tightly bound cytochromes c_1, *b* and aa_3.

The drastically lowered levels of mitochondrial cytochromes may explain why the por^- mutant initially fails to grow on glycerol.

The por^- mutant can regain respiration without regaining porin

When the por^- mutant was plated on solid rich medium containing glycerol as the major carbon and energy source, it slowly started to grow after 3 days at 30°C and had formed normal sized colonies after 5 days. In contrast, wild-type cells formed colonies within 2 days. The growing cells were still completely devoid of porin (not shown) and, upon being transferred to glucose-containing media, lost the ability to grow on glycerol within 1–2 days. Moreover, ~25% of the respiratory-deficient cells initially plated on glycerol eventually started growing. Emergence of growth on glycerol was, thus, not caused by a typical low-frequency suppressor mutation but by some slow adaptation phenomenon.

Once adapted for 5 days, the por^- mutant grew on glycerol at ~1/3–1/2 the rate of the POR^+ parent (Figure 5). As expected, growth of the parent on glycerol was only slightly faster if previous growth had been on that carbon source. Adaptation also led to an increase in the levels of mitochondrial cytochromes (Figure 4, right panels).

Adaptation to growth on glycerol is accompanied by massive accumulation of an 86-kd protein outside the mitochondria

Upon adaptation to glycerol, por^- cells accumulated very large amounts of an 86-kd protein (Figure 6). Judged from the intensity of Coomassie Blue staining, this protein represented up to 20% of the total cellular protein. It was purified and a rabbit antiserum against it was prepared. With the aid of this antiserum, a band of similar mobility (but much lower intensity) could also be detected by immunoblotting in por^- cells grown on glucose. Even lower levels of this protein were detected in POR^+ cells. Upon subcellular fractionation, the 86-kd protein did not cofractionate with mitochondria, but was recovered with a low speed pellet and the microsomal fraction (Figure 7). Preliminary data from sucrose gradients suggested that this did not simply reflect sedimentation of aggregated 86-kd protein with the low and high speed pellets, but true association with the ER or a cell organelle of similar equilibrium density. Whether the 86-kd protein also occurred in the nucleus remained unclear since the low-speed pellet also contained unbroken cells as judged from the abundance of hexokinase in this fraction.

The 86-kd protein was labeled by [^{35}S]cysteine (not shown) and is thus different from a previously described 90-kd yeast heat shock protein which lacks this amino acid (Farrelly and Finkelstein, 1984). The two proteins also differed in overall amino acid composition (K.Suda, unpublished data).

Discussion

The por^- mutant is viable

It has been widely assumed that facilitated diffusion of small molecules across the mitochondrial outer membrane is mediated predominantly, if not exclusively, by a pore composed of porin subunits (Zalman *et al.*, 1980; Mihara *et al.*, 1985; Benz, 1985). This made it reasonable to expect that cells lacking porin would not be viable. The isolation of a porin-deficient viable mutant was thus a major surprise.

Viability of the por^- mutant suggests the existence of other

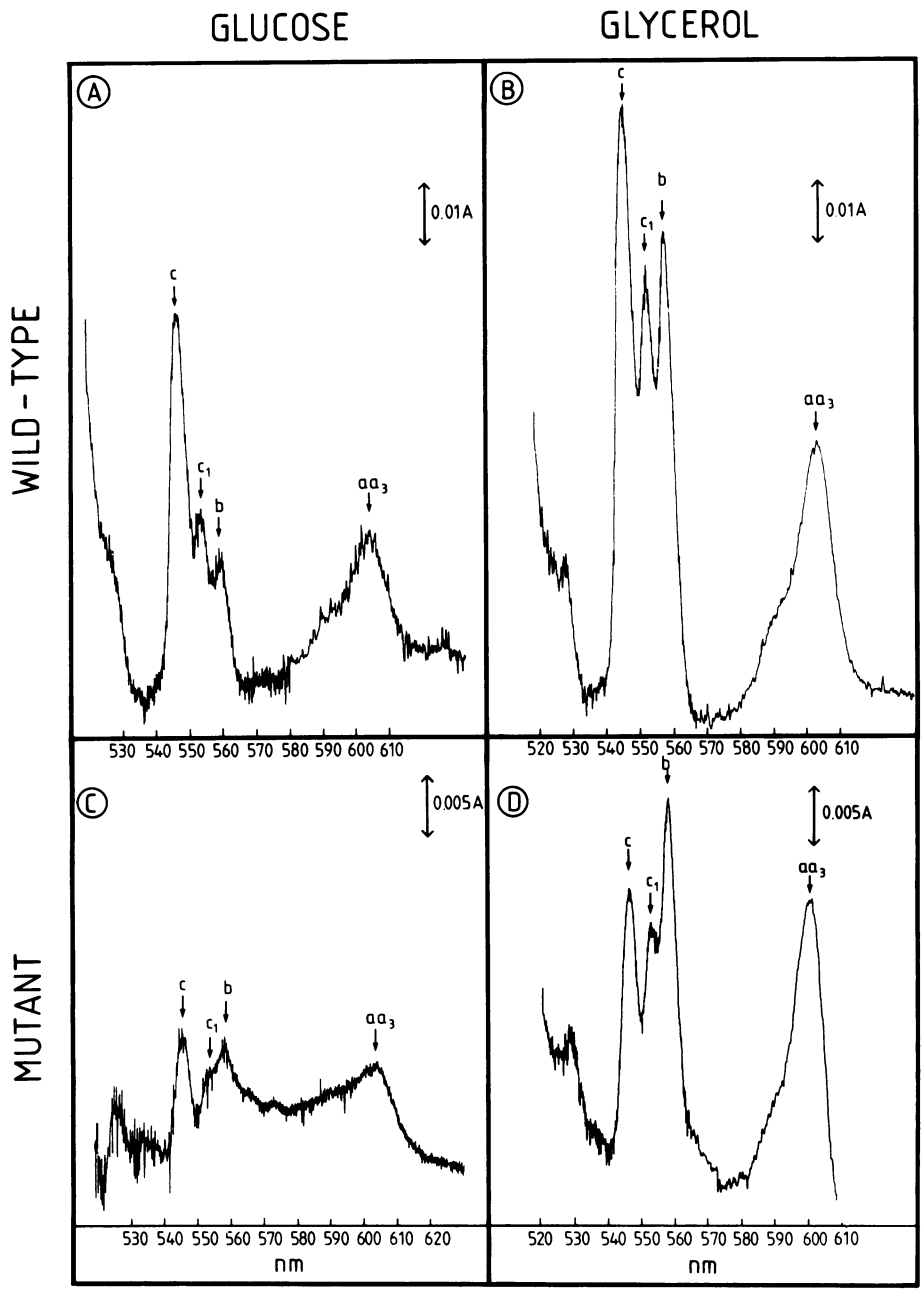

Fig. 4. The por$^-$ mutant is deficient in mitochondrial cytochromes. The POR$^+$ parent strain and the por$^-$ mutant were grown on rich medium containing glucose or, after adaptation, on rich medium containing glycerol. Mitochondria were isolated from the cells and reduced minus oxidized difference spectra were recorded at liquid nitrogen temperature as described (Dowhan *et al.*, 1985) at a band-pass of 2 nm, at a light-path of 2 mm and at the following final concentrations of mitochondrial protein: **A** and **B** at 1.6 mg/ml; **C** and **D** at 2.6 mg/ml. **C** and **D** were recorded at 2-fold higher sensitivity than **A** and **B**.

pathways through which small molecules can traverse the mitochondrial outer membrane. Otherwise it would be difficult to understand how mitochondria could perform their manifold functions without which cells could not survive. For example, the yeast mitochondrial matrix contains key enzymes of amino acid biosynthesis (e.g. Kohlhaw, 1983), yet loss of porin from the cells did not result in additional amino acid requirements. However, it did cause almost complete loss of respiration; this suggests that the major outer membrane pore is essential for the normal passage of one or more intermediate(s) in the synthesis of mitochondrial cytochromes. Possible candidates for such intermediates might be ATP, ADP or intermediates of heme biosynthesis. This point is under investigation. Another possibility would be that the mitochondrial outer membrane in the por$^-$ mutant is leaky *in vivo*, allowing non-specific penetration of small molecules through sites other than pore-forming proteins.

If loss of porin severely limits the passage of charged metabolites through the mitochondrial outer membrane, then intermembrane space enzymes should be latent in por$^-$ mitochondria, in contrast to the situation in POR$^+$ mitochondria (cf. Daum *et al.*, 1982). However, attempts to demonstrate inaccessibility of adenylate kinase to external ADP, or of cytochrome b_2 to external ferricyanide, were unsuccessful (not shown). We suspect that this was caused by the increased fragility of por$^-$ mitochondria (even those from glycerol-adapted cells), but this question needs further study.

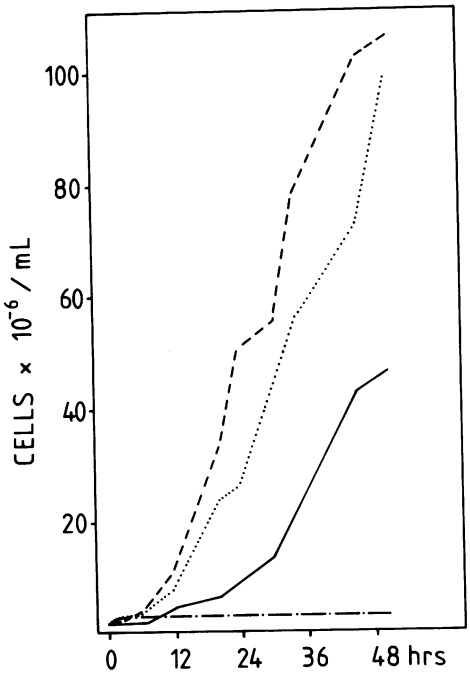

Fig. 5. After adaptation, the por$^-$ mutant grows well on glycerol. Cells were pregrown to the late logarithmic phase in rich medium containing either 2% glucose or 3% glycerol. Aliquots of the precultures were inoculated to 10^6 cells/ml into 100 ml of glycerol-containing rich medium and growth at 30°C was recorded by cell counting. . . . POR$^+$ parent pregrown on glucose; - - - POR$^+$ parent pregrown on glycerol; — · — · — por$^-$ mutant pregrown on glucose; — por$^-$ mutant adapted for 5 days on plates containing glycerol-supplemented rich medium and then pregrown on glycerol.

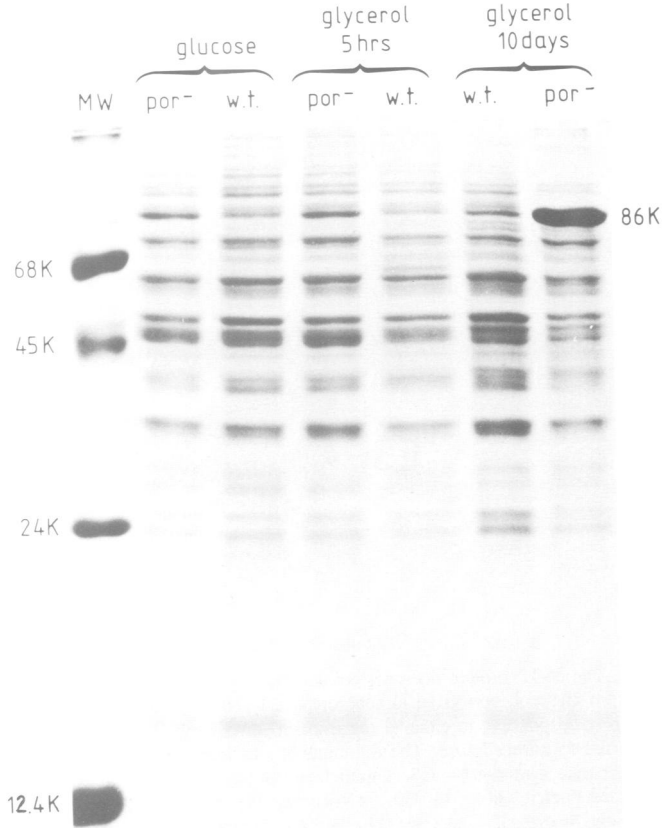

Fig. 6. Massive accumulation of an 86-kd protein accompanies adaptation of por$^-$ cells to growth on glycerol. The POR$^+$ parent (w.t.) and the por$^-$ mutant (por$^-$) were pregrown on rich medium containing glucose and then inoculated into fresh rich medium containing either glucose or glycerol. Growth on glucose was for 5 h; growth on glycerol was for either 5 h or 10 days. Total cell proteins were extracted (Yaffe and Schatz, 1984), 100-μg samples were electrophoresed in an SDS–12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. **Left lane:** mol. wt standards (horse-heart cytochrome *c* 12.4 kd; chymotrypsinogen 24 kd; chicken ovalbumin, 45 kd; bovine serum albumin, 68 kd). The position of the 86-kd protein is marked on the right (86 K).

As the mutant grows nearly as fast as the parent strain on glucose at 30°C, it appears possible that the major pore is only essential for the high-volume traffic of adenine nucleotides across the outer membrane; other minor pores (or leaks) may be sufficient to support the slower passage of other metabolites such as amino acids or Krebs cycle intermediates.

The 86-kd protein

Another unexpected result was that the porin-deficient cells could slowly regain respiration and that this was accompanied by the dramatic accumulation of an extramitochondrial 86-kd protein. It is, of course, possible that this protein is merely one of the many eukaryotic 'stress proteins' (e.g. Farrelly and Finkelstein, 1984) whose accumulation is an indirect consequence of the por$^-$ mutation. However, it is also likely that these two phenomena are mechanistically related even though we have not proved this. The role of the 86-kd protein in por$^-$ cells is not known. It might loosely bind to the mitochondrial surface *in vivo* and thereby increase the efficiency of a minor outer membrane pore, perhaps by concentrating one of the molecules to be transported near the transport site. Indeed, there are data to suggest that the major outer membrane pore also functions as a scaffold for binding cytosolic kinases, thereby allowing these enzymes preferential access to mitochondrially generated ATP (Oestlund *et al.*, 1983). Outer membrane proteins (including porin) could also function as attachment sites for the cytoskeleton (Hirokawa, 1982); loss of a major attachment site in the por$^-$ mutant could perhaps be partly compensated for by increased intracellular levels of the cytoskeletal binding partner. In order to explore the role of the 86-kd protein, we are now cloning its gene.

Materials and methods

Yeast strains, media and plasmids

The *Saccharomyces cerevisiae* strain HR 125-5D (*MAT α gal2 his3 his4 leu2 trp1 ura3*; obtained from D.Allison, Seattle) and a diploid resulting from a cross with the isogenic Mat *a* strain were used as parent strains. Strain SF 747-19D (*MAT α* gal2, his4 leu2 ura3) was a gift from C.Field and R.Schekman, Berkeley. Rich media for yeast growth contained 1% yeast extract, 2% peptone and 2% glucose, or 3% glycerol. Synthetic media (containing the appropriate nutritional supplements for auxotrophs) and semisynthetic media were as decribed (Reid, 1983; Suissa *et al.*, 1984). The yeast–*Escherichia coli* shuttle vector pFL1 (originally from F.Lacroute, Strasbourg) carries the yeast *URA3* gene, the replication origin of the yeast 2 μm plasmid, and the *E. coli* genes for resistance to ampicillin and tetracycline and for replication in *E. coli*.

Recombinant DNA methods

Published procedures were used for yeast transformation, isolation of plasmid DNA or genomic DNA from yeast or *E. coli*, DNA manipulations, and growth of *E. coli* HB101 (as in Suissa *et al.*, 1984). Gene disruption was performed by the one-step method of Rothstein (1983). A 5-kb *Pst*I fragment carrying the disrupted porin gene was integrated into the genome of haploid and diploid yeast cells (HR 125-5D). Owing to the instability of the integrated sequence (Table I) only one haploid and four diploid transformants were found to be por$^-$. A second haploid transformant, which lacked porin in the first screen, recovered and became LEU$^+$ POR$^+$. Since our cloned porin gene appears to have a TY

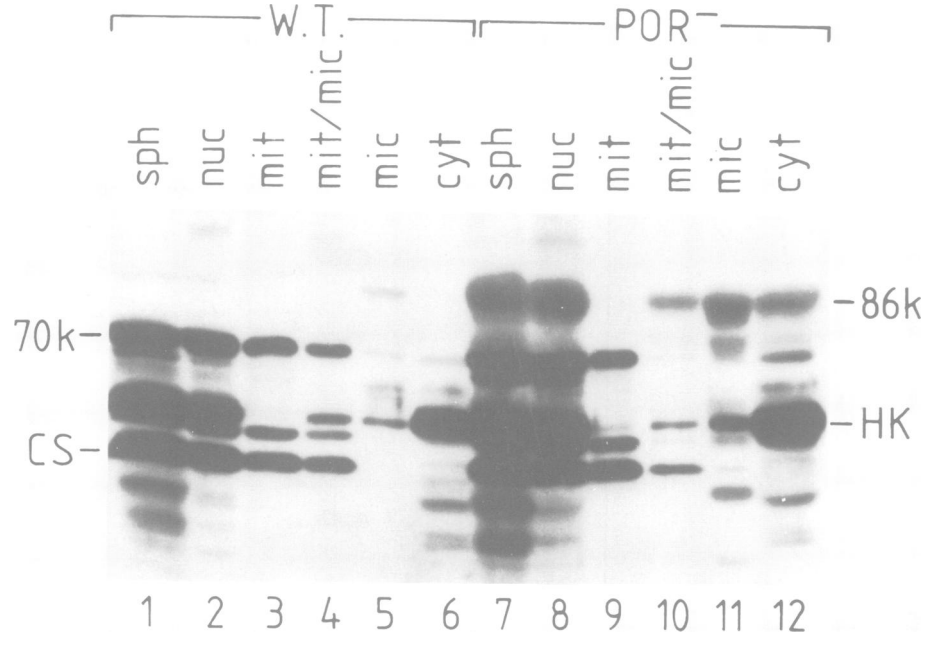

Fig. 7. The 86-kd protein does not cofractionate with mitochondria. Wild-type POR$^+$ **(lanes 1–6** W.T.) or por$^-$ mutant yeast cells **(lanes 7–12** POR$^-$) were grown on glycerol, converted to spheroplasts, lysed by gentle homogenization and subfractionated by differential centrifugation. Amounts of protein from each fraction corresponding to either 5 mg (mutant) or 5.7 mg (W.T.) of wet wt yeast cells were loaded onto an 8% SDS–polyacrylamide gel, electrophoresed and transferred to nitrocellulose. The immunoblot was incubated with antibodies against the 86-kd protein, the 70-k protein of the mitochondrial outer membrane (70 k), citrate synthase (= CS, a mitochondrial matrix marker) and hexokinase (= HK, a cytosolic marker). sph = spheroplasts; nuc = low-speed pellet containing nuclei; mit = 11 950 *g* mitochondrial pellet; mit/mic = 47 800 *g* pellet containing mitochondria and microsomes; mic = 100 000 *g* microsomal pellet; cyt = cytosol. The 100 000 *g* pellet is operationally termed 'microsomes', even though a large part of the fragments of the endoplasmic reticulum may already sediment in the 47 800 *g* pellet. The 100 000 *g* pellet contains most of the ribosomes of the yeast cell (not shown).

element close to its 5′ end (not shown) and since this TY element is also present on the *Pst*I fragment used for gene replacement, the frequent excision of the integrated sequence may be caused by recombination of this element with other related sequences in the yeast genome.

Cell fractionation and biochemical assays

Isolation of mitochondria and other subcellular fractions from yeast spheroplasts was as outlined by Daum *et al.* (1982). Published methods were used for recording low-temperature absorption spectra of mitochondria (Dowhan *et al.*, 1985) for assaying cytochrome *c* oxidase (Mason *et al.*, 1973), cytochrome b_2 (Daum *et al.*, 1982), adenylate kinase (Sottocasa *et al.*, 1967) and fumarase (Racker, 1950). Proteins were extracted from whole yeast cells, separated on SDS–polyacrylamide gels and subjected to immunoblotting as in Yaffe and Schatz (1984). Protein was measured by the BCA assay of Pierce Chemical Co. Yeast cells were pulse-labeled with [^{35}S]methionine as described (Reid, 1983).

Purification of the 86-kd protein

Adapted por$^-$ mutant cells grown in rich medium containing glycerol to the late logarithmic phase (10 g wet wt) were subfractionated as described above, except that the first low-speed spin was omitted. The 100 000 *g* pellet (36 mg protein), which was highly enriched in 86-kd protein, was solubilized in 2 ml 0.1 M NaCl, 20 mM Hepes pH 7.4, 0.2% Triton X-100 and 1.1 ml (20 mg protein) loaded onto a 20-ml DE52 column. After washing the column with 0.1 M NaCl buffer, proteins were eluted with a linear gradient of 0.1–0.5 M NaCl (total volume = 100 ml). Aliquots of each fraction were analysed on SDS–8% polyacrylamide gels for the presence of the 86-kd protein. Fractions containing mainly 86-kd protein were pooled and used directly for immunization of a first rabbit ('native protein'). Alternatively, the 86-kd protein was further purified by electrophoresis on preparative SDS–8% polyacrylamide gels, electroelution from gel slices and dialysis before injection into a second rabbit ('denatured protein').

Preparation of antibodies against the 86-kd protein

Polyclonal antibodies against the 86-kd protein were raised in male Chinchilla rabbits as described previously (Daum *et al.*, 1982); injections were performed intracutaneously with 100–150 μg of pure 86-kd protein mixed with Freund's complete adjuvant.

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