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Proteomic Analysis of the Yeast Mitochondrial Outer Membrane Reveals Accumulation of a Subclass of Preproteins[D]

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Mitochondria consist of four compartments–outer membrane, intermembrane space, inner membrane, and matrix—with crucial but distinct functions for numerous cellular processes. A comprehensive characterization of the proteome of an individual mitochondrial compartment has not been reported so far. We used a eukaryotic model organism, the yeast *Saccharomyces cerevisiae*, to determine the proteome of highly purified mitochondrial outer membranes. We obtained a coverage of ~85% based on the known outer membrane proteins. The proteome represents a rich source for the analysis of new functions of the outer membrane, including the yeast homologue (Hfd1/Ymr110c) of the human protein causing Sjögren–Larsson syndrome. Surprisingly, a subclass of proteins known to reside in internal mitochondrial compartments were found in the outer membrane proteome. These seemingly mislocalized proteins included most top scorers of a recent genome-wide analysis for mRNAs that were targeted to mitochondria and coded for proteins of prokaryotic origin. Together with the enrichment of the precursor form of a matrix protein in the outer membrane, we conclude that the mitochondrial outer membrane not only contains resident proteins but also accumulates a conserved subclass of preproteins destined for internal mitochondrial compartments.

INTRODUCTION

Mitochondria are crucial for numerous functions of virtually every eukaryotic cell, including bioenergetics, apoptosis, and metabolism of amino acids, lipids, and iron (Neupert, 1997; Scheffler, 1999; Newmeyer and Ferguson-Miller, 2003; Green and Kroemer, 2004; Lill and Mühlenhoff, 2005). Many diseases have been linked to mitochondrial dysfunction (Schapira, 2000; Schon, 2000; Steinmetz *et al*., 2002; Wallace, 2005). To date, the most comprehensive proteomic analysis of an entire cell organelle has been performed for mitochondria (Mootha *et al*., 2003; Sickmann *et al*., 2003; Taylor *et al*., 2003; Prokisch *et al*., 2004). In the yeast *S. cerevisiae*, a coverage of ~80% of the mitochondrial proteome was achieved (Sickmann *et al*., 2003; Jensen *et al*., 2004; Prokisch *et al*., 2004; Reichert and Neupert, 2004). These studies indicate that yeast mitochondria contain up to 1000 different proteins, i.e., ~17% of the ~6000 different proteins synthesized in a yeast cell. Because yeast mitochondria synthesize only eight stable proteins encoded by the mitochondrial DNA in their matrix, 99% of all mitochondrial proteins are encoded by nuclear genes and synthesized as precursor proteins on cytosolic ribosomes. These precursor proteins are typically posttranslationally imported into mitochondria and sorted into one of the four mitochondrial compartments: outer membrane, intermembrane space, inner membrane, and matrix (Neupert, 1997; Jensen and Dunn, 2002; Endo *et al*., 2003; Mihara, 2003; Truscott *et al*., 2003; Koehler, 2004). Most preproteins destined for the mitochondrial matrix and several proteins destined for inner membrane or intermembrane space contain amino-terminal presequences that function as targeting signals.

Each of the four mitochondrial compartments harbors specific functions and structures, e.g., the enzymes of the citric acid cycle are located in the matrix, the respiratory chain complexes are present in the inner membrane, factors released from mitochondria during programmed cell death originate from the intermembrane space, and the general entry gate for precursor proteins (translocase of outer membrane [TOM] complex) and the sorting and assembly machinery (SAM) complex are embedded in the outer membrane (Neupert, 1997; Scheffler, 1999; Newmeyer and Ferguson-Miller, 2003; Wiedemann *et al*., 2003; Pfanner *et al*.,

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Abbreviations used: ACN, acetonitrile; BAC, benzyldimethyl-*n*-hexadecylammonium chloride; FA, formic acid; $F_1\beta$, F_0F_1-ATPase subunit β; HA, hemagglutinin; MALDI, matrix-assisted laser desorption ionization; MLR, mitochondrial localization of mRNA; MS/MS, tandem mass spectrometry; SAM, sorting and assembly machinery; SCX, strong cation exchange; TIM, translocase of inner mitochondrial membrane; TOF, time of flight; TOM, translocase of outer mitochondrial membrane.

2004). A systematic proteomic characterization of the individual compartments will be of high importance for a molecular understanding of mitochondrial functions. Da Cruz *et al*. (2003) performed a partial proteomic analysis of enriched mitochondrial inner membranes from mouse liver. However, no comprehensive analysis of an individual mitochondrial compartment has been reported so far; thus, our knowledge about the composition and functional relevance of the mitochondrial compartments is limited.

Here, we performed a comprehensive proteomic analysis of the outer membrane of yeast mitochondria. Our experimental strategy involved the following steps. 1) Because a high purity of an isolated mitochondrial compartment is an essential prerequisite for a proteomic analysis, we selected outer membrane vesicles, which of all four mitochondrial compartments of *S. cerevisiae* are best accessible to a selective purification (Alconada *et al*., 1995; van Wilpe *et al*., 1999; Meisinger *et al*., 2001). Protocols for high-purity isolation of mitochondria and outer membrane vesicles were combined in a successive manner to yield pure outer membranes. 2) To avoid the limitations of individual separation and detection methods, we used several different approaches for separation of proteins and tryptic peptides as well as for the analysis by mass spectrometry, in parallel. 3) Unexpected protein localization results were subjected to an integrative analysis by comparison to a genome-wide study of mRNA targeting to mitochondria and evolutionary relationships of mitochondrial proteins (Marc *et al*., 2002). These combined approaches yielded important new insight into mitochondrial dynamics and biogenesis.

MATERIALS AND METHODS

Isolation of Mitochondrial Outer Membrane Vesicles

Yeast wild-type strain YPH499 (Sikorski and Hieter, 1989) was grown in YPG medium (1% (wt/vol) yeast extract, 2% (wt/vol) bactopeptone, and 3% (wt/vol) glycerol) at 30°C until an optical density of 2. A crude mitochondrial fraction (P_{12}) was obtained by differential centrifugation as described previously (Daum *et al*., 1982; Meisinger *et al*., 2000). This fraction was further purified via two three-step sucrose gradients, yielding highly pure mitochondria (Meisinger *et al*., 2000; Sickmann *et al*., 2003). For isolation of outer membranes, purified mitochondria (100 mg of protein) were diluted in swelling buffer (5 mM potassium phosphate, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated on ice for 20 min at a protein concentration of 2–4 mg/ml. After treatment with a glass-Teflon potter (15–20 strokes), the homogenate was loaded on top of a discontinuous sucrose gradient [1 ml 60%, 4 ml 32%, 1 ml 15% (wt/vol) sucrose] in EM buffer [10 mM 3-(*N*-morpholino)propanesulfonic acid MOPS, pH 7.2, and 1 mM EDTA)]. After centrifugation in a swing-out rotor (1 h; 134,000 × *g*; 2°C), outer membranes were recovered from the 15–32% interface, and the sucrose concentration was adjusted to 50% with a 70% (wt/vol) sucrose/EM solution. This sample was then loaded onto the bottom of a centrifuge tube and overlaid with 5 ml of 32% (wt/vol) sucrose/EM and 1.5 ml of EM buffer. After flotation at 240,000 × *g* at 2°C overnight, purified outer membrane vesicles were collected from the 0–32% interface, diluted fivefold with EM buffer, and pelleted via centrifugation at 160,000 × *g* for 30 min (Alconada *et al*., 1995; Meisinger *et al*., 2001). Membranes were resuspended in EM buffer and stored in aliquots at −80°C.

Separation of Outer and Inner Membrane Vesicles

Mitochondrial pellets (2–5 mg of protein) were resuspended in 20 mM HEPES/KOH, pH 7.4, 0.5 mM EDTA, and 1 mM PMSF and incubated for 30 min on ice at a concentration of 1 mg/ml. After adjustment to a final sucrose concentration of 0.45 M and further incubation for 10 min on ice, the sample was sonified with a Branson Sonifier 250 (duty cycle 80%) for 90 s. After a clarifying spin at 20,000 × *g* for 15 min, the supernatant was centrifuged at 200,000 × *g* for 45 min. The pellet, containing membrane vesicles, was resuspended in 400 μl of loading buffer (5 mM HEPES/KOH, pH 7.4, 10 mM KCl, and 1 mM PMSF) and layered on top of a discontinuous sucrose gradient (1.5 ml of 1.6 M sucrose, 5.5 ml of 1.35 M sucrose, 2.5 ml of 1.1 M sucrose, and 1.5 ml of 0.85 M sucrose in 5 mM HEPES/KOH, pH 7.4, and 10 mM KCl). After centrifugation at 134,000 × *g* and 2°C for 16 h, fractions (750 μl) were collected and analyzed by SDS-PAGE and Western blotting using polyvinylidene diflouride (PVDF) membranes (Millipore, Billerica, MA) and the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Quantification of immunoreactive bands was performed with the NIH Image program.

Mitochondrial Surface Fraction

Purified mitochondria were incubated at a protein concentration of 1 mg/ml in SEM buffer (10 mM MOPS, pH 7.2, 250 mM sucrose, 1 mM EDTA) containing 20 μg/ml trypsin for 15 min on ice. After centrifugation for 15 min at 20,000 × *g*, the supernatant, containing the mitochondrial surface fraction, was separated by SDS-PAGE. Gel slices (1 mm) were excised and subjected to in-gel digestion followed by mass spectrometry based peptide identification as described for two-dimensional benzyldimethyl-*n*-hexadecylammonium chloride (2D-BAC)/SDS-PAGE (see below).

2D-BAC/SDS-PAGE

Outer membrane vesicles were centrifuged in EM buffer at 100,000 × *g* and 4°C for 1 h. Pellets were resuspended in 5 μl of 1× BAC sample buffer (3.75 M urea, 125 mM BAC, 5% glycerol, 0.025% pyronin Y, and 60 mM dithiothreitol [DTT]) and incubated at 60°C for 10 min. First dimensions were carried out either on 10, 12, or 15% slab gels overlaid with 4% stacking gels (Zahedi *et al*., 2005) for 20 min at 4°C and 30 mA/gel followed by 60 min at 60 mA/gel. Afterward, whole sample lanes were excised, equilibrated three times for 10 min with 100 mM Tris-HCl, pH 6.8, and incubated in 3× SDS sample buffer containing 60 mM DTT for 10 min. Lanes were transferred on 4–20, 10, or 12% Tris-glycine SDS gels. Second dimensions were carried out at 4°C for 15 min at 15 mA/gel and then for 40 min at 50 mA/gel. Subsequently, gels were stained according to Shevchenko *et al*. (1996). Visualized protein spots were excised. Washing of spots and in-gel digestion were accomplished according to Shevchenko *et al*. (1996) with slight modifications. Dried protein spots were incubated with 12.5 ng/μl trypsin (Sequencing grade modified; Promega, Madison, WI) in 50 mM NH_4HCO_3. Digestion was carried out for 8 h at 37°C.

Peptides were extracted, depending on the following mass spectrometric (MS) method, in a three-step (nano-liquid chromatography-tandem mass spectrometry [LC-MS/MS]) or in a two-step (matrix-assisted laser desorption ionization [MALDI]-MS) procedure. For nano-LC-MS/MS analysis, peptides were extracted twice with 10 μl of 5% formic acid (FA) each and once with 10 μl of 5% FA, 50% acetonitrile (ACN). Extracts were combined, and the organic content was reduced under vacuum to improve compatibility to reversed-phase chromatography. For MALDI-MS, peptides were extracted once with 10 μl of 0.1% trifluoroacetic acid, concentrated using μC_{18} ZipTips (Millipore) according to the manufacturer's instructions, and eluted directly onto a MTP 384 polished steel target plate (Bruker Daltonik, Bremen, Germany).

Multidimensional Liquid Chromatography (MDLC)

Outer membrane vesicles in 20 μl of EM buffer were digested using 0.5 μg of trypsin (5000 U/mg) in 20 μl of 25 mM NH_4HCO_3 for 8 h at 37°C. The sample volume was reduced to 6 μl in a vacuum centrifuge, and the solution was acidified using 5% FA. In the first dimension, the tryptic peptide mixture was separated by strong cation exchange chromatography (PL-SCX; 300-μm inner diameter [ID], 150-mm length, 1000-Å pore size, 8-μm particle size, custom packed) using a linear binary gradient (solvent A: 20 mM KH_2PO_4, pH 3.0; solvent B: 20 mM KH_2PO_4, 0.25 mM NaCl, 25% ACN, pH 5.5). Although increasing the overall peptide recovery, the use of an organic modifier renders online nano-LC-MS/MS coupling impossible (Wagner *et al*., 2003). Therefore, minute-by-minute fractions were collected, the organic solvent was removed under vacuum, and each sample was analyzed in the second dimension by nano-LC-MS/MS separately.

Nano-LC-MS/MS

Nano-LC-MS/MS analyses were accomplished either on a LCQ Deca XPplus (Thermo Electron, Dreieich, Germany), a Qstar XL (Applied Biosystems, Darmstadt, Germany), or a Qtrap 4000 (Applied Biosystems) mass spectrometer directly coupled to an UltiMate nano-LC system (Dionex, Idstein, Germany). Samples were online-desalted and preconcentrated using a C18 PepMap trapping column (300-μm ID, 1-mm length, 100-Å pore size, 5-μm particle size; Dionex) and subsequently separated on a C18 PepMap main column (75-μm ID, 150-mm length, 100-Å pore size, 3-μm particle size; Dionex) using a linear binary gradient (solvent A: 0.1% FA; solvent B: 0.1% FA, 84% ACN) at a flow rate of 250 nl/min according to Mitulovic *et al*. (2003, 2004). Full MS scans from 400 to 1600 *m/z* were recorded, and the four (LCQ Deca XPplus, Qtrap 4000) or three (Qstar XL) most intensive peptide ions were subjected to further fragmentation considering a dynamic exclusion.

MALDI-Time of Flight (TOF)-MS/MS

MALDI-time of flight analyses were accomplished on an Ultraflex TOF/TOF (Bruker Daltonik) in reflector mode after external calibration using a custom-made peptide standard. Additionally, acquired spectra were internally calibrated using tryptic autoproteolysis signals at 842.5094 *m/z*, 2211.104 *m/z*, and 2283.180 *m/z*.

Data Interpretation

To exclude consideration of false positive protein hits derived from search algorithms, only proteins, which were identified by both MASCOT as well as SEQUEST and of which at least two independent fragment ion spectra were validated manually, were taken into account. An in-house software solution named resDB was used for filtering, grouping, and viewing the Mascot result files obtained from the MS/MS data. resDB is a database based solution running on the relational database system PostgreSQL featuring import and export of MS/MS result files, including a spectra archive. The user application features viewing single results as well as grouping of one or more MDLC runs that usually yield Mascot result files of more than half a gigabyte in size, which cannot be handled by the viewer coming with Mascot. For internal peptide hit scoring, we normalized each single Mascot score with its corresponding significance threshold. Grouped protein hits were scored according to the count of unique peptides, spectra per peptide and exact protein sequence coverage. The application additionally features the presentation of peptide results, including the corresponding MS/MS spectrum and the theoretical peptide spectrum as well as a direct link to our internal protein sequence database seqDB that presents sequence coverage and peptide locations.

The significance of a single peptide hit obtained by using Mascot can be calculated from the Mascot score S_m and the internal significance p in conjunction with the corresponding score threshold s_p according to the following equations:

$$S_m = -10 \cdot \log_{10} S \tag{1}$$

$$S_p = 10 \cdot \log_{10}\left(\frac{m}{20\,p}\right) \xrightarrow{p = 0.05} 10 \cdot \log_{10} m \tag{2}$$

with m being the average of the internal scores S of a single Mascot run.

Given another significance level y, a new threshold S_y can be calculated according to

$$s_y = 10 \cdot \log_{10}\left(\frac{m}{20\,y}\right) = 10 \cdot \log_{10}\left(\frac{m}{20\,px}\right) = 10\left(\log_{10}\left(\frac{1}{x}\right) + \log_{10}\left(\frac{m}{20\,p}\right)\right) \xrightarrow{y = px} s_p + 10 \cdot \log_{10}\left(\frac{p}{y}\right) \tag{3}$$

Therefore, the significance level of a single Mascot score $S = s_y$ can be calculated as follows, if the original threshold s_p and significance p are available –the default value of p is 0.05.

$$s_y = s_p + 10 \cdot \log_{10}\left(\frac{p}{x}\right) \Leftrightarrow \frac{s_y - s_p}{10} = \log_{10}\left(\frac{1}{x}\right) \Leftrightarrow 10^{\frac{s_y - s_p}{10}} = \frac{1}{x} = \frac{p}{y} \Leftrightarrow y = \frac{p}{10^{\frac{s_y - s_p}{10}}} \tag{4}$$

According to Eq. 4, we calculated the significance of each peptide hit as the probability for a false positive.

For generation of a complete peptide list and calculation of the sequence coverage, only peptides with a Mascot score above the threshold $s_p + 10$ (MLDC and 2D-BAC/SDS-PAGE samples) were taken into account, corresponding to a probability of 0.005 for a false positive. Molecular weights and isoelectric points were calculated (Skoog and Wichmann, 1986) according to the sequences deposited in the *Saccharomyces* Genome Database (Cherry *et al*., 1997).

RESULTS

Identification of Proteins in Purified Mitochondrial Outer Membrane Vesicles

The experimental strategy is outlined in Figure 1A. *S. cerevisiae* mitochondria were isolated by differential centrifugation. This crude mitochondrial fraction was virtually free of the cytosolic marker protein phosphoglycerate kinase and the nuclear marker Nsp1, but it contained significant contaminations from the endoplasmic reticulum (Sss1), vacuole (alkaline phosphatase [ALP]), and a small fraction of peroxisomes (Pex13) (Figure 1B) (Meisinger *et al*., 2000; Sickmann *et al*., 2003). By applying two successive sucrose gradients, highly pure mitochondria were obtained that were also virtually free of the marker proteins for the endoplasmic reticulum, vacuole, and peroxisomes (Figure 1B) (Sickmann *et al*., 2003). By gentle mechanical forces applied to the mitochondria, outer membrane vesicles were sheared off and separated from the remaining mitochondrial compartments by successive sedimentation and flotation steps. The resulting outer membrane vesicles (Figure 1C, lane 2) were devoid of marker proteins for the intermembrane space, inner membrane, and matrix (Figure 1C, lane 4). In addition, we prepared a mitochondrial surface fraction (MSF) by a treatment of isolated mitochondria with trypsin (Sickmann *et al*., 2003). The trypsin treatment degraded the surface-exposed receptor Tom70 but did not disrupt the outer membrane barrier as indicated by a limited clipping of the outer membrane-integrated channel Tom40 (Hill *et al*., 1998) and the protection of the intermembrane space-exposed proteins translocase of inner mitochondrial membrane (Tim) 13 and Tim23 (Figure 1D).

Although 2D-gel electrophoresis has been widely used for proteomic analysis, many proteins, in particular membrane proteins, escape detection by this method. For example, in the comprehensive proteomic analysis of yeast mitochondria with 749 identified different proteins, only 109 different proteins (15%) were identified by an extensive 2D-analysis (Sickmann *et al*., 2003). We thus used a 2D-analysis optimized for membrane proteins with the detergent BAC in the first dimension and SDS in the second dimension (Zahedi *et al*., 2005). By nano-LC-MS/MS and MALDI-MS/MS, we identified 36 different proteins from purified outer membrane vesicles (Figure 2 and Supplemental Table 1). However, only 31% of the known outer membrane proteins were identified by this approach.

We thus subjected purified outer membranes to an extensive treatment with trypsin and separated the peptide mixture by SCX-chromatography. Nano-LC-MS/MS led to the identification of 112 different proteins (Table 1) with a coverage of ~85% of all known mitochondrial outer membrane proteins, including integral and peripheral membrane proteins (Supplemental Table 2). In comparison to our previous analysis of the mitochondrial proteome (Sickmann *et al*., 2003), 27 additional proteins were identified (Supplemental Table 3). The additional proteins were mostly of low abundance (Ghaemmaghami *et al*., 2003), including Mdm10, Mdm34, Ubp16, and three subunits of the tRNA splicing endonuclease complex (Sen2, Sen34, and Sen54) (Yoshihisa *et al*., 2003), indicating a high sensitivity of the detection system applied.

Thirteen of the 112 proteins have not been studied previously, i.e., were encoded by an *S. cerevisiae* open reading frame (ORF) without gene name. The other 99 proteins were encoded by named genes, although a number of these proteins had been previously localized to other cellular compartments or, in particular, to one of the three internal mitochondrial compartments, intermembrane space, inner membrane, and matrix (see below). In the experiments shown in Supplemental Figure S1, we studied several proteins, which were reported to be located in other cellular compartments in some studies: Erg9 and Erg6 that are involved in sterol synthesis, a member of the Rho family (Rho1), Pho88, and the alcohol acyl transferase Eht1 (Leber *et al*., 1998; Kumar *et al*., 2002; Huh *et al*., 2003). We also included two proteins of unknown function and localization: Yor285w, a conserved protein with high homology to the *Drosophila melanogaster* heat-shock protein 67B2 (Pauli *et al*., 1988), and Hfd1 (Ymr110c; see below). Localization in the mitochondrial outer membrane was analyzed by different means. 1) Membrane vesicles of purified mitochondria were generated, and outer and inner membrane vesicles were separated by sucrose density gradient centrifugation. We determined whether the proteins carrying a hemagglutinin

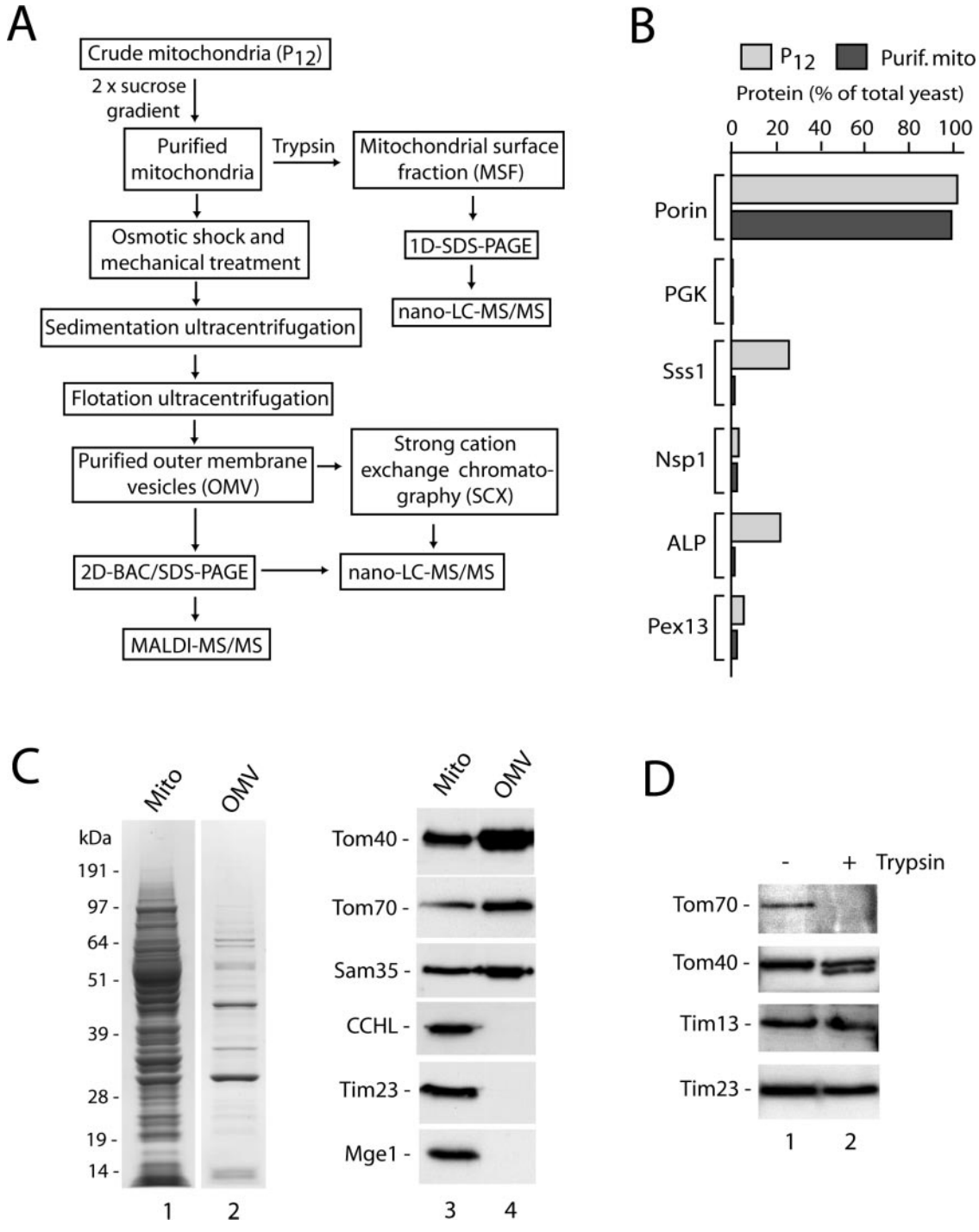

Figure 1. Experimental strategy and purity of mitochondrial fractions from *S. cerevisiae*. (A) Flow chart showing purification of mitochondrial outer membranes and mitochondrial surface fraction followed by MS analysis. (B) Isolation of highly purified mitochondria. Crude mitochondrial fractions (P_{12}) were purified twice via sucrose gradients, yielding purified mitochondria essentially devoid of marker proteins from other cell compartments. Equal-volume aliquots from mitochondria and total yeast extracts were separated by SDS-PAGE, followed by Western blotting and immunodecoration. Immunoreactive bands were quantified and the value for total yeast extract of each protein was set to 100%. Porin, mitochondrial outer membrane protein; PGK, phosphoglycerate kinase (cytosol); Sss1, subunit of Sec61 translocation complex (endoplasmic reticulum); Nsp1, subunit of nuclear pore complex; ALP, vacuolar ALP; Pex13, peroxin13. (C) Purification of outer membrane vesicles. Purified mitochondria and outer membrane vesicles were separated by SDS-PAGE and then either stained with Coomassie brilliant blue (lanes 1 and 2) or transferred onto PVDF membranes followed by immunodecoration (lanes 3 and 4). Tom40, Tom70, subunits of the preprotein translocase of the outer membrane; Sam35, subunit of the sorting and assembly machinery of the outer membrane; CCHL, cytochrome *c* heme lyase of the intermembrane space; Tim23, subunit of the presequence translocase of the inner membrane; Mge1, soluble matrix protein. (D) Generation of the MSF. Highly purified mitochondria were incubated with or without trypsin (20 μg/ml) in SEM buffer for 15 min on ice. After pelleting, the mitochondria were separated by SDS-PAGE and analyzed by Western blotting and immunodecoration, whereas the supernatant (MSF) was subjected to nano-LC-MS/MS analysis (Supplemental Table 4).

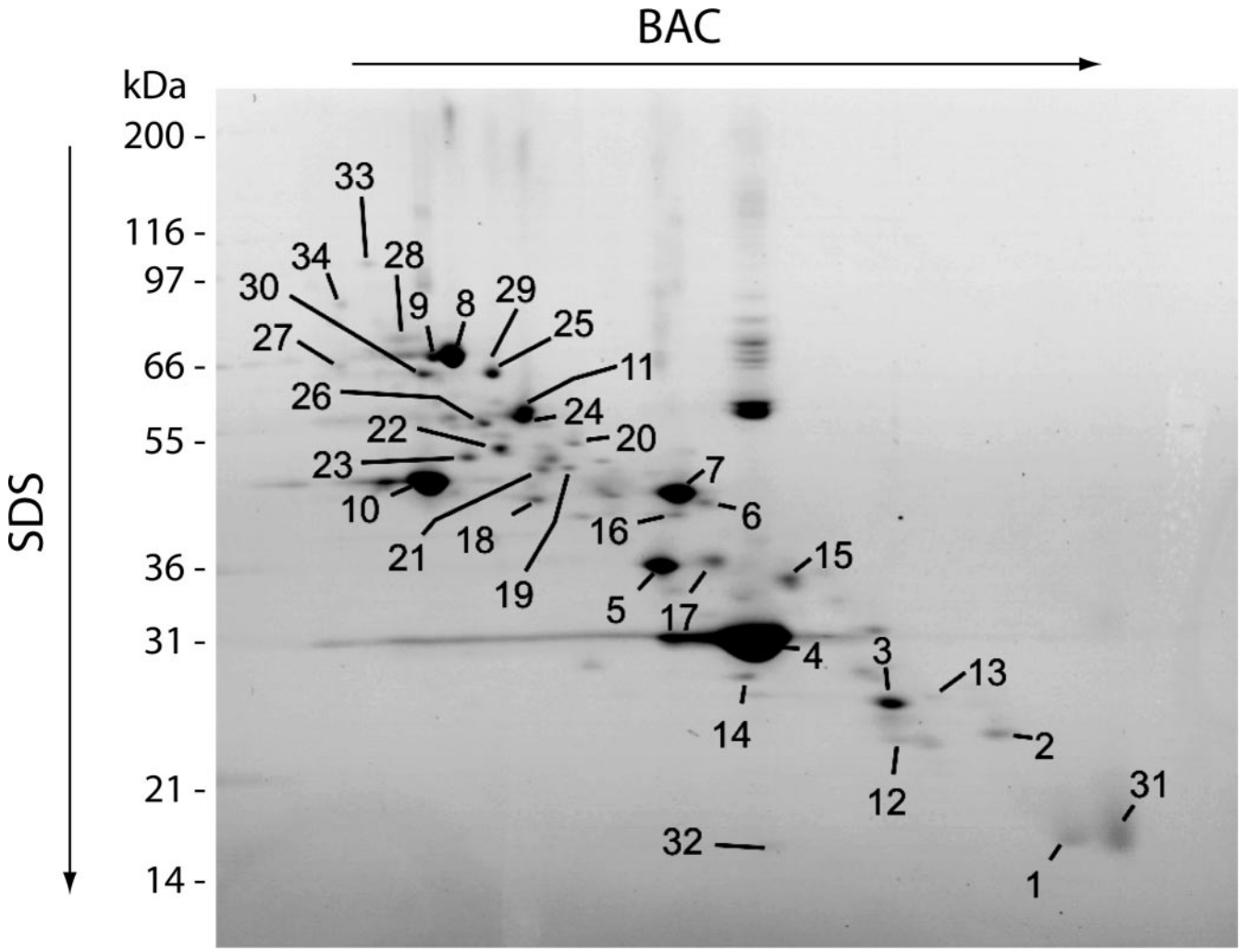

Figure 2. 2D-gel analysis of purified mitochondrial outer membranes. Purified outer membranes (~30 μg of protein) of *S. cerevisiae* mitochondria were separated by BAC-PAGE in the first dimension followed by SDS-PAGE in the second dimension. Coomassie-stained spots were excised and analyzed either by nano-LC-MS/MS or MALDI-MS/MS as described in *Materials and Methods*. Thirty-six different proteins were identified and listed in Supplemental Table 1.

(HA)-tag cofractionated with the outer membrane marker Tom40 or with the inner membrane marker Tim23 (Supplemental Figure S1, A–C). Erg9, Erg6, and Pho88 each cofractionated exclusively with the outer membrane. (2) Authentic, i.e., nontagged, Yor285w and Rho1 were synthesized in the presence of [^{35}S]methionine/cysteine in reticulocyte lysate and imported into isolated yeast mitochondria. The import was independent of the presence or absence of a membrane potential $\Delta\psi$ across the inner mitochondrial membrane (Supplemental Figure S1D, lanes 2, 3, 7, and 8), and the imported proteins remained largely accessible to proteinase K added to the mitochondria (Supplemental Figure S1D, lanes 4, 5, 9, and 10). Thus, the proteins showed the two characteristic features of mitochondrial outer membrane proteins (Wiedemann *et al*., 2005). Localization of Yor285w and Rho1 at mitochondria was also reported in independent studies (Sickmann *et al*., 2003; Ohlmeier *et al*., 2004). 3) HA-tagged Hfd1 and Eht1 were accessible to protease added to isolated mitochondria (Supplemental Figure S1E; see below; and Figure 3). Thus, each protein analyzed behaved like a typical outer membrane protein, confirming the findings of the mass spectrometry analysis.

The Yeast Homologue of Human Fatty Aldehyde Dehydrogenase (FALDH) Is Mainly Located in the Mitochondrial Outer Membrane

The open reading frame YMR110c coded for a new protein of 59.9 kDa with significant similarity to human fatty aldehyde dehydrogenase, including two predicted hydrophobic sequences of sufficient length to function as transmembrane segments (Figure 3A). Mutations in the gene for human FALDH have been shown to be responsible for the Sjögren–Larsson syndrome, an inherited neurocutaneous disorder with mental retardation, spasticity, and ichthyosis (De Laurenzi *et al*., 1996; Rizzo *et al*., 2001).

Different views have been reported on the subcellular localization of mammalian fatty aldehyde dehydrogenase, including microsomes, mitochondria, and peroxisomes (Miyauchi *et al*., 1993; Kelson *et al*., 1997). Yeast Ymr110c, now termed Hfd1 for homologue of fatty aldehyde dehydrogenase, has been found in so-called lipid particles, intracellular lipid-rich structures consisting of a hydrophobic core with a surrounding phospholipid monolayer and a limited set of proteins (Athenstaedt *et al*., 1999; Huh *et al*.,

Table 1. List of all proteins identified in the mitochondrial outer membrane fraction separated by strong cation exchange chromatography (SCX) followed by reversed phase chromatography coupled to ESI-MS (nano-LC-MS/MS)

Gene	Protein	ORF	Sequence coverage (%)	Predicted molecular mass (kDa)	Predicted isoelectric point	MSF	MLR	2D-BAC/SDS-PAGE
ATP2	F_1F_0-ATPase complex, $F_1\beta$ subunit	YJR121w	42	54.7	5.5	X	98.2	X
PDB1	Pyruvate dehydrogenase (lipoamide) β chain precursor	YBR221c	17	40.0	5.2	X	97.5	
COR1	Ubiquinol-cytochrome *c* reductase 44K core protein	YBL045c	14	50.2	6.8	X	96.8	X
ALD4	Mitochondrial aldehyde dehydrogenase	YOR374w	45	56.7	6.3	X	96.2	
NDI1	NADH-ubiquinone-6 oxidoreductase (homologue to human outer membrane protein AMID)	YML120c	6	57.2	9.4		96.0	
MDH1	Malate dehydrogenase, mitochondrial	YKL085w	34	35.6	8.5	X	95.2	
ATP3	F_1F_0-ATPase complex, $F_1\gamma$ subunit	YBR039w	32	34.3	9.3	X	95.0	
MCR1	Cytochrome b_5 reductase	YKL150w	64	34.1	8.7	X	95.0	X
PDA1	Pyruvate dehydrogenase complex E1-α subunit	YER178w	23	46.3	8.3	X	95.0	
SDH2	Succinate dehydrogenase iron–sulfur protein subunit	YLL041c	18	30.2	9.1		94.8	
VPS1	Member of the dynamin family of GTPases	YKR001c	25	78.7	7.7		94.0	
MIA40	Essential mitochondrial protein required for import and assembly of intermembrane space proteins	YKL195w	6	44.5	4.5	X	92.0	
STE24	Zinc metallo-protease	YJR117w	18	52.3	7.7		91.7	
HSP60	Heat-shock protein-chaperone, mitochondrial	YLR259c	42	60.7	5.2	X	90.0	
TOM70	Mitochondrial outer membrane import receptor subunit	YNL121c	57	70.0	5.2	X	90.0	X
KGD1	2-Oxoglutarate dehydrogenase complex E1 component	YIL125w	27	114.3	6.8	X	89.4	X
GUT2	Glycerol-3-phosphate dehydrogenase, mitochondrial	YIL155c	48	72.3	8.0	X	89.4	X
CYB2	Lactate dehydrogenase cytochrome b_2	YML054c	14	65.5	8.6	X	89.0	X
FAA1	Long chain fatty acid-CoA ligase	YOR317w	47	77.8	7.5		89.0	X
OM45	Protein of the outer mitochondrial membrane	YIL136w	66	44.5	8.6	X	88.6	X
SDH1	Succinate dehydrogenase flavoprotein	YKL148c	20	70.2	7.1		87.8	X
NCP1	NADPH-cytochrome P450 reductase	YHR042w	3	76.7	5.0		87.0	
GSF2	Protein involved in glucose repression	YML048w	7	45.8	6.6		87.0	
AFG1	Member of the AFG1-like ATPase family	YEL052w	6	58.3	8.7		86.0	
COR2	Ubiquinol-cytochrome *c* reductase 40-kDa chain II	YPR191w	35	40.4	7.8	X	83.8	X
TIM44	Subunit of the presequence translocase associated motor complex (PAM complex)	YIL022w	16	48.8	9.4		83.0	
ILV5	Ketol-acid reducto-isomerase	YLR355c	19	44.3	9.1	X	82.7	
BNA4	Kynurenine 3-mono oxygenase	YBL098w	33	52.4	8.6		82.0	X
NDE1	Mitochondrial NADH dehydrogenase that catalyzes cytosolic NADH oxidation	YMR145c	28	62.7	9.3	X	81.7	
MDM34	Protein essential for maintaining wild-type mitochondrial morphology	YGL219c	5	51.9	9.1		81.3	
ERG6	*S*-Adenosyl-methionine Δ-24-sterol-C-methyltransferase	YML008c	38	43.4	5.5		80.0	
SCM4	Suppressor of Cdc4 mutation	YGR049w	17	20.1	8.8		77.3	
ERG9	Farnesyl-diphosphate farnesyltransferase	YHR190w	7	51.7	5.6		77.0	
CBR1	Cytochrome *b* reductase	YIL043c	28	36.2	9.3		75.0	
SEL1	Adaptor for Cdc48p-mediated protein degradation via the ubiquitin-proteasome pathway	YML013w	13	66.7	5.3		74.0	
FUN14	Protein of unknown function	YAL008w	10	22.0	10.5		74.0	
ILV2	Acetolactate synthase, first step in valine and isoleucine biosynthesis pathway	YMR108w	9	74.9	8.6		71.8	
SSC1	Mitochondrial heat-shock protein 70 (HSP70)	YJR045c	25	70.6	5.5	X	70.0	
NCA2	Control of mitochondrial synthesis of Atp6p and Atp8p	YPR155c	27	70.8	6.4		65.0	X
—	Electron-transferring flavoprotein, β chain	YGR207c	15	28.7	8.6		63.3	
UIP4	Protein of unknown function, weak similarity to *Xenopus* protein xlgv7	YPL186c	17	34.2	4.4		61.5	
EHT1	Alcohol acyl transferase	YBR177c	34	51.2	7.6		61.2	X
LSP1	Strong similarity to YGR086c	YPL004c	36	38.0	4.6	X	60.5	
VPS21	Small GTP-binding protein	YOR089c	41	23.1	5.3		60.0	
DPM1	Dolichyl-phosphate β-D-mannosyltransferase	YPR183w	25	30.3	7.7		60.0	

Table 1. (Continued)

Gene	Protein	ORF	Sequence coverage (%)	Predicted molecular mass (kDa)	Predicted isoelectric point	MSF	MLR	2D-BAC/SDS-PAGE
YPT31	GTP-binding protein of the Rab family	YER031c	53	24.5	5.0		58.0	
HSP10	Heat-shock protein-chaperonin, mitochondrial	YOR020c	28	11.4	9.0	X	57.0	
MGM1	Protein that mediates mitochondrial inheritance and is required for mitochondrial outer membrane fusion, member of dynamin family of GTPases	YOR211c	22	101.5	8.1		55.5	
IML2	Protein with a role in stability of artificial minichromosomes	YJL082w	16	82.5	6.2		54.0	
SAM35	Subunit of the mitochondrial sorting and assembly machinery (SAM complex)	YHR083w	13	37.4	6.7		53.5	
PTH2	Aminoacyl-tRNA hydrolase; homologue to mammalian mitochondrial BIT1 (Bcl-2 inhibitor of transcription)	YBL057c	43	23.1	5.3		52.0	
GEM1	Conserved tail-anchored outer mitochondrial membrane GTPase which regulates mitochondrial morphology	YAL048c	12	75.1	5.7		51.0	
—	Hypothetical protein	YPR098c	9	17.7	9.7		51.0	X
—	Hypothetical protein, similarity to hypothetical *Escherichia coli* and *Caenorhabditis elegans* proteins	YER004w	44	25.1	9.3		50.0	X
—	Similarity to *Emericella nidulans* cysteine synthase	YGR012w	57	42.8	8.5		50.0	X
PIL1	Strong similarity to hypothetical protein YPL004c	YGR086c	38	38.3	4.5	X	49.0	X
—	Hypothetical protein, similarity to aldehyde dehydrogenase	YMR110c	33	59.9	6.3		48.0	X
TOM20	Mitochondrial outer membrane import receptor subunit, 20 kDa	YGR082w	36	20.3	5.6	X	46.0	X
ATP5	F_1F_0-ATPase complex, OSCP subunit	YDR298c	47	22.8	9.6		38.5	
YJU3	Protein containing an α- or β-hydrolase fold domain, has weak similarity to human monoglyceride lipase	YKL094w	28	35.5	8.5		38.0	
MIR1	Mitochondrial phosphate carrier - member of the mitochondrial carrier family	YJR077c	32	32.8	9.4		37.0	
ATP15	F_1F_0-ATPase complex, F_1 epsilon subunit	YPL271w	84	6.7	9.8	X	36.3	
RHO1	GTP-binding protein of the Rho subfamily of Ras-like proteins	YPR165w	13	23.1	6.0		35.0	
SEC4	Secretory vesicle-associated Rab GTPase essential for exocytosis	YFL005w	48	23.5	6.6		34.0	
YPT32	Small GTP-binding protein essential for Golgi function	YGL210w	42	24.5	5.3		33.0	
ZEO1	Mid2p-interacting protein, modulates the PKC1–MPK1 cell integrity pathway	YOL109w	41	12.6	5.4	X	32.7	
CYC1	Cytochrome *c* isoform 1	YJR048w	55	12.1	9.5		31.2	X
—	Hypothetical protein	YGR266w	7	81.1	6.4		30.0	
FIS1	Protein involved in mitochondrial division	YIL065c	46	17.7	9.2		28.5	X
YPT7	GTP-binding protein of the RAB family	YML001w	28	23.0	4.9		28.5	
POR2	Putative mitochondrial porin (voltage-dependent anion channel)	YIL114c	11	31.1	9.7		28.0	
—	Hypothetical protein, similarity to *D. melanogaster* heat-shock protein 67B2	YOR285w	45	15.4	5.9		27.5	
FZO1	Protein involved in mitochondrial fusion and maintenance of the mitochondrial genome	YBR179c	32	97.7	6.6		26.5	
TOM7	Subunit of the preprotein translocase of the mitochondrial outer membrane (TOM complex)	YNL070w	27	6.9	8.3		24.0	
AYR1	1-Acyldihydroxyacetone-phosphate reductase	YIL124w	49	32.8	9.2		20.5	X
POR1	Mitochondrial porin (voltage-dependent anion channel)	YNL055c	64	30.4	7.7	X	19.0	X
GTT1	Glutathione transferase	YIR038c	21	26.8	6.2		18.7	
UGO1	Outer membrane component of the mitochondrial fusion machinery	YDR470c	21	57.4	6.6		13.5	
MRPS17	Protein of the mitochondrial small ribosomal subunit	YMR188c	26	27.6	9.6		9.0	
TSC10	3-Ketosphinganine reductase	YBR265w	15	36.0	5.9			
MHR1	Involved in mitochondrial homologous DNA recombination	YDR296w	20	26.9	9.5			
MSP1	Intramitochondrial sorting protein	YGR028w	38	40.3	5.5			X

Table 1. (Continued)

Gene	Protein	ORF	Sequence coverage (%)	Predicted molecular mass (kDa)	Predicted isoelectric point	MSF	MLR	2D-BAC/SDS-PAGE
—	Hypothetical protein, strong similarity to molybdopterin-converting factor homologue YKL027w	YHR003c	38	48.8	6.4			X
YSC83	Hypothetical protein, strong similarity to *Saccharomyces douglasii* YSD83	YHR017w	24	44.2	9.1			
TOM72	Protein translocase 72-kDa component of the outer membrane of mitochondria	YHR117w	41	71.8	5.8			X
—	Hypothetical protein, strong similarity to hypothetical protein YHR199c	YHR198c	26	36.5	9.1			
TIM8	Mitochondrial intermembrane space protein involved in transport of proteins into the inner membrane	YJR135w-a	24	9.8	5.2			
—	Hypothetical protein, similarity to *E. coli* molybdopterin-converting factor chlN	YKL027w	62	50.3	8.9			X
SAC1	Polyphosphoinositide phosphatase, required for transport of ATP into ER	YKL212w	30	71.1	7.3			X
MMM1	Protein required for mitochondrial shape and structure	YLL006w	5	48.6	5.5			
ALO1	D-Arabinono-1,4-lactone oxidase	YML086c	37	59.5	6.4			X
SAM37	Subunit of the mitochondrial sorting and assembly machinery (SAM complex)	YMR060c	17	37.4	6.9			
YIM1	Mitochondrial inner membrane protease	YMR152w	30	41.6	7.6			
TOM40	Subunit of the preprotein translocase of the mitochondrial outer membrane (TOM complex)	YMR203w	14	42.0	5.3			X
TOM22	Subunit of the preprotein translocase of the mitochondrial outer membrane (TOM complex)	YNL131w	30	16.8	4.0			X
PET123	Mitochondrial ribosomal protein, small subunit	YOR158w	19	36.0	9.8			
—	Hypothetical protein	YOR228c	7	34.0	10.0			
UBP16	Ubiquitin-specific protease	YPL072w	22	56.9	9.2			
TOM5	Subunit of the preprotein translocase of the mitochondrial outer membrane (TOM complex)	YPR133w-a	30	6.0	8.1			
—	Hypothetical protein	YJL133c-a	36	7.7	11.4			
ERG27	3-Keto sterol reductase, ERGosterol biosynthesis	YLR100w	18	39.7	8.3			
—	Conserved hypothetical protein	YDR381c-a	17	12.8	9.8			
MDM10	Subunit of the mitochondrial SAM complex; required for normal mitochondrial morphology and inheritance	YAL010c	13	56.2	6.8			
PDH1	Hypothetical protein, similarity to *Bacillus subtilis* mmgE protein	YPR002w	18	57.6	9.2			
MDV1	Protein involved in mitochondrial division and mitochondrial fission	YJL112w	8	80.0	5.4			
MMR1	Phosphorylated protein of the mitochondrial outer membrane	YLR190w	9	54.8	7.5			
SEN2	tRNA splicing endonuclease, β subunit	YLR105c	17	44.1	8.6			
SAM50	Subunit of the mitochondrial sorting and assembly machinery (SAM complex)	YNL026w	13	54.5	8.7			
TIM9	Mitochondrial intermembrane space protein involved in transport of proteins into the inner membrane	YEL020w-a	29	10.2	8.4			
SEN54	tRNA splicing endonuclease, α subunit	YPL083c	21	54.6	9.1			
SEN34	tRNA splicing endonuclease, γ subunit	YAR008w	20	31.3	7.1			
NUC1	Nuclease with both DNase and RNase activity, major nuclease of mitochondria	YJL208c	11	37.2	9.0			

Proteins that were also identified in the MSF or via 2D-BAC/SDS-PAGE are indicated (X). MLR, value for mitochondrial localization of mRNA. The identified peptides are listed in Supplemental Table 7. —, no assigned gene name.

2003). Currently, 37 different yeast proteins have been found in lipid particles (according to the Yeast Proteome Database; www.incyte.com). We identified five of those 37 proteins in the mitochondrial outer membrane proteome: Ayr1, Eht1, Erg6, Faa1, and Hfd1 (Ymr110c). The same five proteins of lipid particles were also found in the proteomic analyses of total yeast mitochondria (Sickmann *et al*., 2003; Prokisch *et al*., 2004), whereas most other proteins of lipid particles were

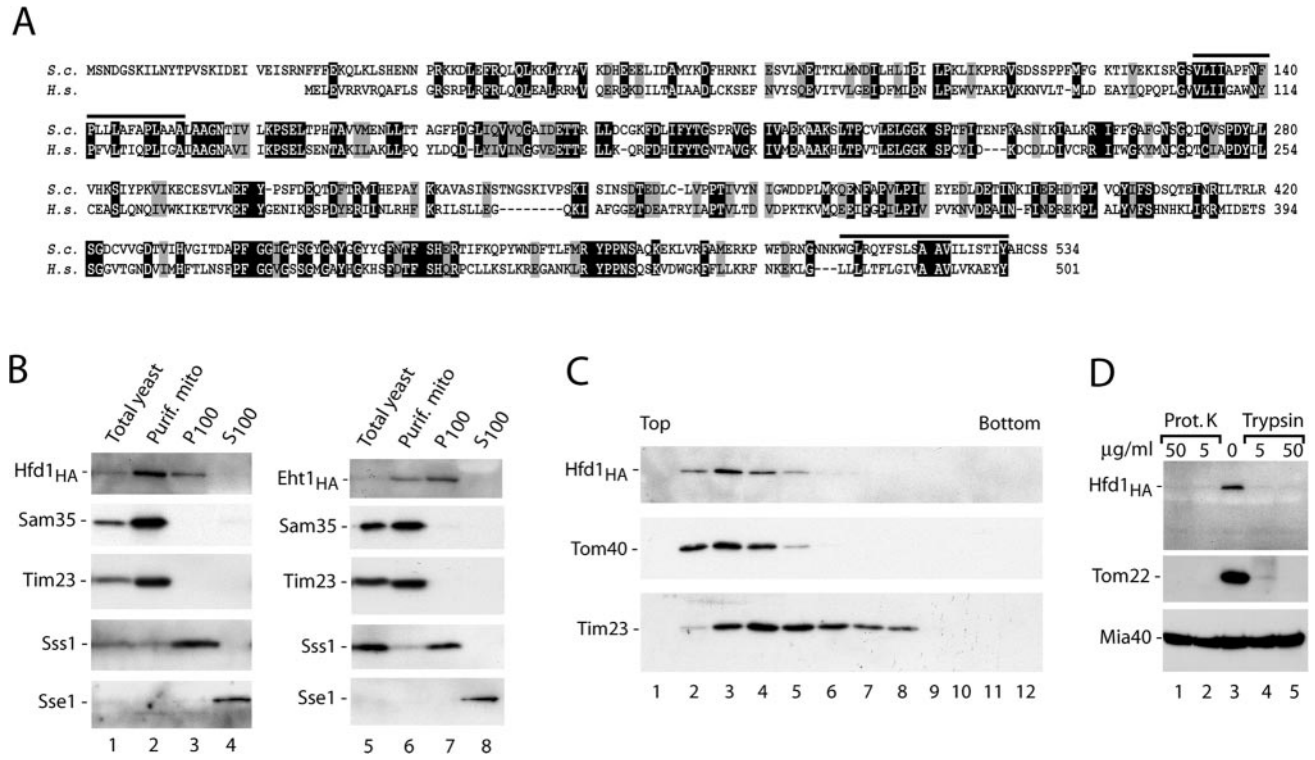

Figure 3. The homologue of human fatty aldehyde dehydrogenase is mainly located in the mitochondrial outer membrane. (A) Comparison of the predicted primary structures of *S. cerevisiae* Hfd1, encoded by the open reading frame YMR110c (*S.c.*), and the human fatty aldehyde dehydrogenase FALDH (*H.s.*). Black, identical residues; gray, similar residues; bars, predicted hydrophobic transmembrane segments found by the HMMTOP program (Tusnady and Simon, 2001). (B) Subcellular fractionation of yeast strains, containing HA-tagged versions of Hfd1 or Eht1 (Open Biosystems, Brussels, Belgium), according to Meisinger *et al*. (2000), followed by SDS-PAGE and Western blot analysis. Equal amounts of proteins were loaded. The mitochondrial fraction was purified by sucrose gradient purification. P_{100}, S_{100}, pellet, and supernatant of 100,000 $\times$ *g* centrifugation. Sss1, subunit of the Sec61 protein translocase (endoplasmic reticulum); Sse1, cytosolic heat-shock protein 70. (C) $Hfd1_{HA}$ is located in the outer membrane. Separation of outer and inner membrane vesicles from $Hfd1_{HA}$ mitochondria on a sucrose gradient was performed as described in *Materials and Methods*. Fractions were analyzed by SDS-PAGE and immunodecoration using antisera against HA, the outer membrane protein Tom40, and the inner membrane protein Tim23. (D) Mitochondrial Hfd1 is accessible to proteases. Highly purified mitochondria (50 μg of protein) isolated from the $Hfd1_{HA}$ strain were resuspended in 100 μl of SEM buffer containing either proteinase K or trypsin and incubated for 15 min on ice. Proteinase K was inhibited by adding 2 mM PMSF, and trypsin was blocked by a 30-fold excess of soybean pancreatic trypsin inhibitor. After washing with SEM, mitochondria were separated on SDS-PAGE and analyzed by Western blotting and immunodecoration.

not found in mitochondria in any of these proteomic studies. It is thus unlikely that the mitochondrial preparations were simply contaminated with lipid particles. A comparison of the localization data in the literature suggested the possibility of a dual/multiple localization for at least some of these proteins (McCammon *et al*., 1984; Leber *et al*., 1998; Kumar *et al*., 2002; Huh *et al*., 2003; Sickmann *et al*., 2003; Prokisch *et al*., 2004). We performed a cellular fractionation and found HA-tagged Hfd1 in both the purified mitochondrial fraction and the P_{100} fraction, whereas the mitochondrial marker proteins Sam35 (outer membrane) and Tim23 were only found in the mitochondrial fraction (Figure 3B), supporting the view of a dual localization of Hfd1. For comparison, we determined the localization of HA-tagged Eht1 and found it also both in the mitochondrial and P_{100} fractions (Figure 3B). Although Eht1 was enriched in the P_{100} fraction, Hfd1 was mainly located in the highly purified mitochondria (Figure 3B), indicating that mitochondria represented the major cellular location of Hfd1.

The submitochondrial localization of Hfd1 was analyzed by separation of outer and inner membrane vesicles on a sucrose gradient. Hfd1 cofractionated with Tom40 and not with Tim23 (Figure 3C). When isolated mitochondria were treated with proteinase K or trypsin, Hfd1 was degraded like the outer membrane receptor Tom22 (Figure 3D). The mitochondrial intermembrane space protein Mia40 (Chacinska *et al*., 2004; Naoé *et al*., 2004; Terziyska *et al*., 2005) was protected against the proteases, demonstrating that the outer membrane barrier of the mitochondria was not damaged by the protease treatment. Thus, $Hfd1_{HA}$ is exposed on the mitochondrial surface. The localization of $Hfd1_{HA}$ and $Eht1_{HA}$ in the mitochondrial outer membrane was independent of the growth of the cells on nonfermentable medium (Figure 3) or fermentable medium (Supplemental Figure S1, E). Together, these results show that Hfd1 is mainly located in the mitochondrial outer membrane.

Correlation between Preprotein Accumulation at the Mitochondrial Outer Membrane and mRNA Targeting to Mitochondria

Surprisingly, the list of proteins that were identified in the purified outer membrane vesicles (Table 1) contains numerous proteins that are known to be located in one of the three internal mitochondrial compartments. The high purity of the outer membrane vesicles shown in Figure 1C argued against the simple possibility that the outer membranes used here

were contaminated with all other mitochondrial compartments. To obtain further evidence, we prepared a mitochondrial surface fraction by a mild treatment with trypsin that did not disrupt the outer membrane barrier (see above; Figure 1D). The peptides identified in the mitochondrial surface fraction belonged to 49 different proteins (Supplemental Table 4). Besides five outer membrane proteins that were accessible to trypsin (like outer membrane TOM-receptors), however, the mitochondrial surface fraction contained mainly proteins known to reside in one of the three internal mitochondrial compartments (Supplemental Table 4). Moreover, several of the proteins from internal mitochondrial compartments were seen as Coomassie-stained spots on the 2D-BAC/SDS-PAGE (Figure 2, Table 1, and Supplemental Table 1), excluding that they were only present in tiny negligible amounts. It should be noted that only one of the 49 proteins identified in the mitochondrial surface fraction had previously been located to a cellular location outside mitochondria, underscoring the purity of the mitochondria used. The nonmitochondrial protein is the large abundant plasma membrane protein Pma2. Pma2 is known to be easily accessible to tryptic digest and mass spectrometric analysis, leading to identification of tiny amounts, and thus represents a frequent contaminant in proteomic analyses (Washburn *et al*., 2001; Sickmann *et al*., 2003). We analyzed the distribution of the proteins found in the mitochondrial surface fraction according to their abundance in the cell reported in the yeast database of Ghaemmaghami *et al*. (2003). The proteins were present in the complete range of abundance without any detectable bias toward proteins of low abundance or high abundance (Supplemental Table 5).

We performed systematic database searches to find a specific characteristic of those proteins that are residents of internal mitochondrial compartments but are also found in outer membrane vesicles and mitochondrial surface fractions. Unexpectedly, we found a striking relation to a genome-wide analysis of mRNAs targeted to yeast mitochondria. Marc *et al*. (2002) had used yeast DNA microarrays to identify mRNAs found in association with mitochondria. Based on the ratio of mRNA presence in mitochondria-bound polysomes versus free cytosolic polysomes, they defined a value of mitochondrial localization of mRNA (MLR) from 1 (no mitochondrial association) to 100 (mitochondrial association). Marc *et al*. (2002) compared two populations of mitochondrial proteins: conserved proteins with prokaryotic homologues and proteins that are only found in eukaryotes. The majority of prokaryote-derived mitochondrial proteins possessed MLR values above 70, whereas the majority of eukaryote-derived mitochondrial proteins displayed MLR values below 55. Marc *et al*. (2002) listed a group of 25 proteins with the highest MLR values of all mitochondrial proteins analyzed. Although only one of those 25 MLR-top scorers was a resident outer membrane protein (Mcr1, which is located both in the outer membrane and the intermembrane space; Hahne *et al*., 1994), 17 of these 25 proteins were found in the outer membrane/mitochondrial surface fraction.

We thus performed a systematic comparison of the MLR values for different populations of yeast proteins. When all yeast proteins analyzed in the genome-wide MLR study are used, a broad distribution of MLR values with a peak at MLR values of 40–60 is observed (Figure 4A) (Marc *et al*., 2002). By analyzing the proteins found in the yeast mitochondrial proteome by Sickmann *et al*. (2003), a shift to higher MLR values was observed with a peak at 70–90 (mainly prokaryote-derived mitochondrial proteins), although numerous mitochondrial proteins with low MLR values were also observed (eukaryote derived) (Figure 4B)

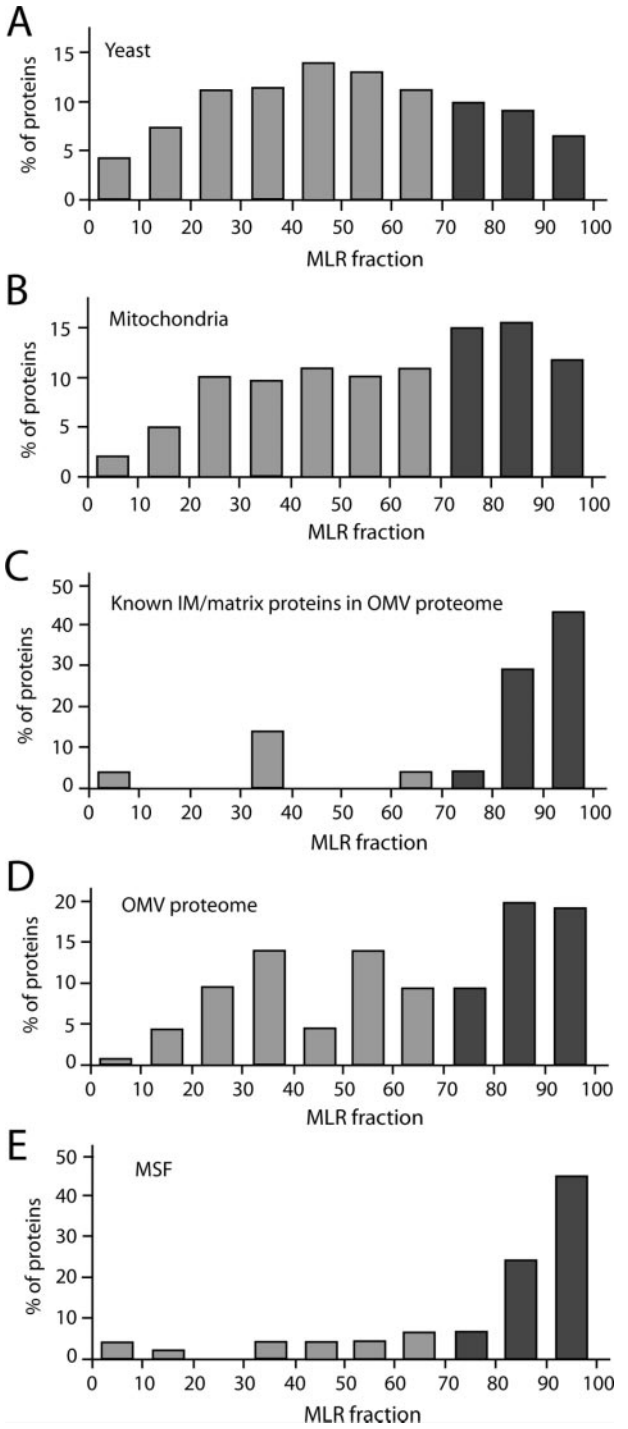

Figure 4. Comparison of the mitochondrial outer membrane proteome to a genome-wide analysis of mRNAs targeted to mitochondria. MLR values of 70 or higher (dark columns) indicate a high probability of mRNA localization to the mitochondrial environment (values are accessible at www.biologie.ens.fr/lgmgml/publication/mitoloc/) (Marc *et al*., 2002). Shown are the MLR fractions (MLR values grouped in blocks of 10: ≥90, ≥80, ≥70, and so on) calculated for all proteins of the classes below for which MLR values are available. (A) Proteins predicted from the *S. cerevisiae* genome (n = 3190). (B) Mitochondrial proteome (n = 422; Sickmann *et al*., 2003). (C) Known inner membrane (IM) and matrix proteins found in the outer membrane proteome (n = 21; Table 1). (D) Proteome of outer membrane vesicles (OMV) (n = 79; Table 1). (E) MSF (n = 47; Supplemental Table 4).

(Marc *et al*., 2002). When all known inner membrane and matrix proteins found in the outer membrane proteome were analyzed, most of them displayed MLR values above 80 (Figure 4C and Table 1). This highly asymmetric MLR distribution is significantly different from that of the total outer membrane proteome (Figure 4D). Similarly, the tryptic peptides found in the mitochondrial surface fraction were mostly derived from proteins with MLR values above 80 (Figure 4E and Supplemental Table 4). Thus, proteins with MLR values above 80 found at the mitochondrial outer membrane/surface are highly enriched in prokaryote-derived mitochondrial proteins that are residents of internal mitochondrial compartments.

We wondered whether these results based on mRNA targeting could be correlated with results on the protein level. A possible hint was the identification of presequence segments in tryptic peptides analyzed in the proteome of yeast mitochondria (Sickmann *et al*., 2003). We asked whether the outer membrane vesicles contained presequence forms of mitochondrial proteins. Indeed, the intermembrane space protein cytochrome b_2, which was detected as Coomassie-stained protein in the 2D-BAC/SDS-PAGE (Figure 2 and Supplemental Table 1), yielded peptides from its presequence (Figure 5A). Cytochrome b_2 is processed in two steps and thus contains two different kinds of presequence segments, an amino-terminal matrix targeting signal and a subsequent inner membrane-sorting signal (Hartl *et al*., 1987; Glick *et al*., 1992; Schwarz *et al*., 1993; Gärtner *et al*., 1995). An internal peptide of each of these presequence segments was identified (Figure 5A), indicating that the intact precursor form was accumulated in outer membrane vesicles. We used antibodies specific for F_0F_1-ATPase subunit β ($F_1\beta$), which is associated with the matrix side of the inner membrane, to study a possible accumulation of the precursor form in outer membrane vesicles. By sonication of mitochondria, we generated outer and inner membrane vesicles and separated them by sucrose density gradient centrifugation. Mature $F_1\beta$ fractionated with the inner membrane protein Tim23 as expected (Figure 5B). In addition, we observed the precursor form of $F_1\beta$ in the Western blot analysis. The precursor protein cofractionated with the outer membrane protein Tom40, demonstrating that the precursor was indeed accumulated at the outer membrane.

We subjected the 13 new proteins found in the outer membrane proteome to three different prediction programs for amino-terminal presequences, which direct proteins to internal mitochondrial compartments. Three of the proteins, Yjl133c-a, Yhr198c, and Ygr207c, were predicted to contain a characteristic mitochondrial presequence in all three programs (Supplemental Table 6; Nakai and Horton, 1999; Guda *et al*., 2004; Small *et al*., 2004), indicating that they belong to the group of internal proteins accumulated as precursors at the outer membrane. For six of the remaining 10 new proteins, MLR values are available (Table 1) (Marc *et al*., 2002). Each of these MLR values is below 55, suggesting that these proteins without predicted presequence belong to the eukaryote-derived mitochondrial proteins and are mostly resident outer membrane proteins. Indeed, the biochemical analysis of two of these proteins, Hfd1 and Yor285w, confirmed their predominant localization in the mitochondrial outer membrane (see above).

The experiments showing an accumulation of preproteins destined for internal mitochondrial compartments at the outer membrane were performed upon cell growth on nonfermentable medium, i.e., under conditions of high mitochondrial activity and high synthesis rates for mitochondrial proteins. A possible hypothesis for the accumulation of preproteins at the outer membrane would be an overflow of the presequence import pathway that is mainly used by the conserved class of proteins. To test this hypothesis, we prepared mitochondrial surface fractions by treating isolated mitochondria with a low concentration of trypsin upon cell growth at two different conditions, nonfermentable medium and fermentable medium. Silver staining of SDS-gels revealed significant differences in the intensity of a number of bands containing proteins/protein fragments (Supplemental Figure S2A). In most cases, the stained bands were stronger when mitochondria were prepared after cell growth on nonfermentable medium. Using nano-LC-MS/MS, we identified 16 different proteins from internal mitochondrial compartments in these bands. Remarkably, 15 of these proteins possessed high MLR values (Supplemental Figure S2A), indicating a specific enrichment of this subclass of proteins at the outer membrane under nonfermentable conditions. We controlled this finding by Western blot analysis for the matrix protein Hsp60. The isolated mitochondria contained similar amounts of Hsp60 upon growth under nonfermentable and fermentable conditions, but the mitochondrial surface fraction contained a strongly increased level of Hsp60 at nonfermentable conditions (Supplemental Figure S2B), confirming the result obtained by mass spectrometry. Resident proteins of the outer membrane, such as Sam35 and Tom70, were present in similar amounts at both growth conditions in mitochondria as well as in the mitochondrial surface fraction (trypsin-generated fragments of Sam35 and Tom70) (Supplemental Figure S2B). Thus, growth conditions that require a high activity of mitochondria lead to an accumulation of proteins, which possess high MLR values and are residents of internal mitochondrial compartments, at the mitochondrial surface.

DISCUSSION

We have performed a comprehensive analysis of the proteome of mitochondrial outer membranes by a multidimensional approach and obtained a coverage of ~85% of all known outer membrane proteins. This represents the highest coverage obtained for a proteome of any mitochondrial compartment to date. The approach led to the identification of numerous proteins of low abundance, indicating that the combination of sensitive detection methods with the strong enrichment of proteins in a highly pure submitochondrial fraction yields high sensitivity.

The database reported here represents a rich source for the analysis of new functions of the mitochondrial outer membrane. Interesting examples include a subclass of proteins that are also found in lipid particles and likely possess a dual localization in the cell. A detailed analysis of a new protein (yeast open reading frame YMR110c) previously located to lipid particles revealed a dual localization with a preferential presence in the mitochondrial outer membrane. We termed this protein Hfd1 because it shows significant homology to human fatty aldehyde dehydrogenase that has been shown to be responsible for the inherited Sjögren–Larsson syndrome (De Laurenzi *et al*., 1996; Rizzo *et al*., 2001).

The outer membrane proteome includes 12 GTP-binding proteins (Table 1), suggesting that the mitochondrial outer membrane harbors numerous regulatory processes. Many of these proteins belong to the Ras superfamily. In the case of Ypt7, the mammalian homologue Rab32 was found to be specifically associated with mitochondria (Alto *et al*., 2002). For Gem1, a member of the Rho family, an outer membrane localization and a regulatory function in mitochondrial mor-

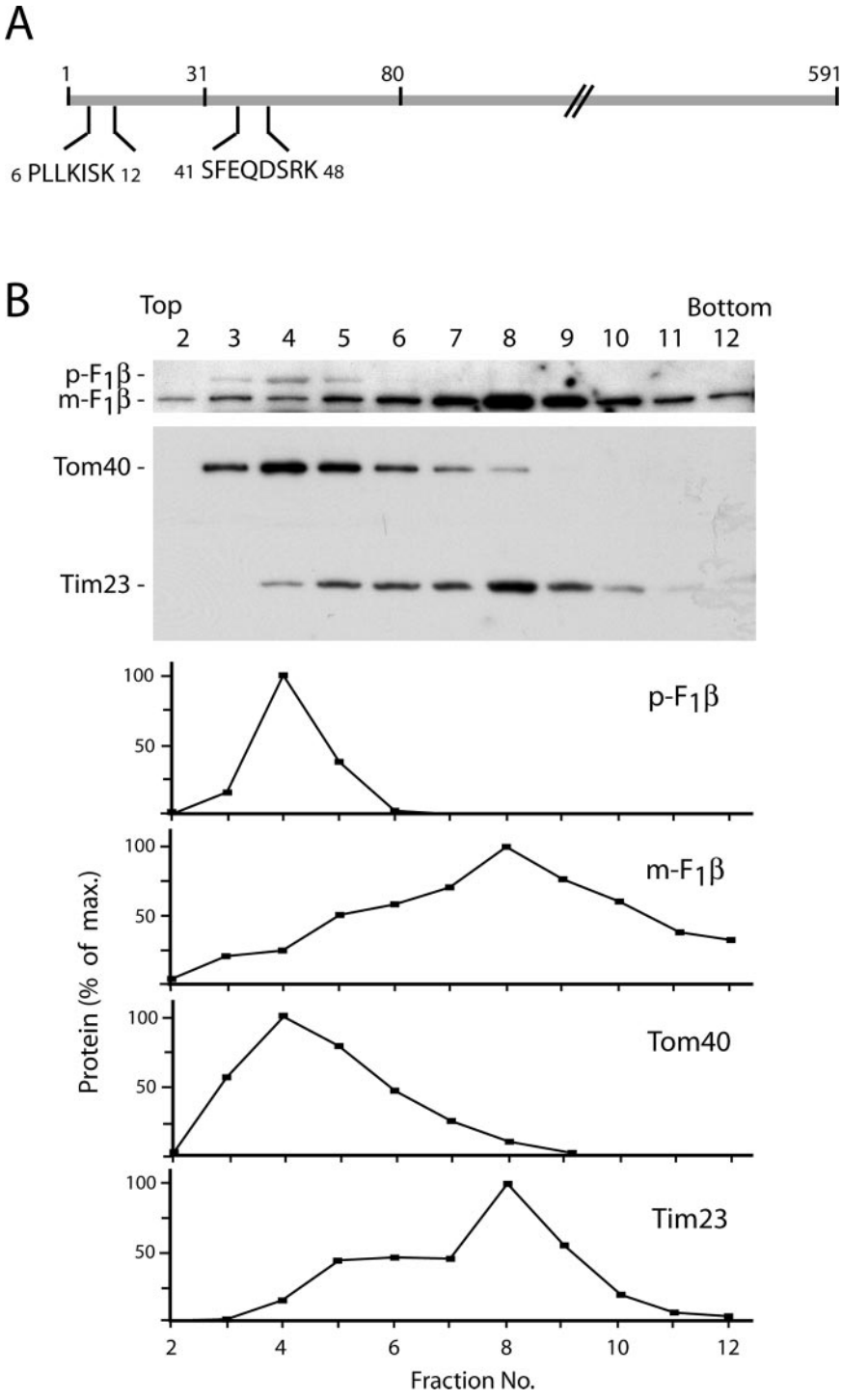

Figure 5. Accumulation of the precursor form of a cleavable mitochondrial protein in the mitochondrial outer membrane. (A) Two presequence peptides of cytochrome b_2 identified from the 2D-BAC/SDS-PAGE of purified outer membranes vesicles (Figure 2). Numbers indicate amino acid residues. The processing of cytochrome b_2 occurs in two steps, after residue 31 and after residue 80. (B) The precursor form of $F_1\beta$ migrates in the outer membrane fraction. Outer and inner membrane vesicles from wild-type yeast mitochondria were separated on a sucrose gradient, and fractions were analyzed by SDS-PAGE and Western blotting. Immunoreactive bands were quantified and the maximal value was set to 100% for each protein. p-$F_1\beta$, precursor form; m-$F_1\beta$, mature protein.

phology was recently reported (Frederick *et al*., 2004). We showed for Rho1, another member of the Rho family identified in the outer membrane proteome, that the in vitro-synthesized protein specifically associates with mitochondria. Another interesting group is formed by proteins involved in ubiquitin-dependent protein degradation, including a ubiquitin-regulatory protein (Sel1) and the ubiquitin-specific protease Ubp16, which was recently found to reside in the mitochondrial outer membrane (Kinner and Kölling, 2003). Two of the new proteins, Ykl027w and Yhr003c, contain motifs that are found in ubiquitin-activating enzymes. We also identified homologues of mammalian proteins involved in apoptosis, such as Pth2 (a homologue of human Bit1; Jan *et al*., 2004), Ndi1 (a homologue of AMID; Wu *et al*., 2002), and Yer004w, which is homologous to mammalian HTATIP2, a protein acting in tumor suppression and apoptosis (Hodges *et al*., 2002). Moreover, we identified three subunits of the tRNA splicing endonuclease complex (Sen2, Sen34, and Sen54) in the purified outer membrane. Indeed, it was recently shown that this complex functions outside of the nucleus in maturation of pre-tRNA (Takano *et al*., 2005) and shows a preferential location at

mitochondria (Yoshihisa *et al*., 2003). To confirm the results of the mass spectrometry analysis, we determined the cellular localization of several identified proteins, in particular proteins that were previously localized to other cellular compartments and some new proteins. In each case, we found that the proteins were indeed located at the mitochondrial surface. Our findings are in agreement with the view that a significant number of mitochondrial proteins show a dual localization in the cell, i.e., are located in mitochondria and an additional cellular compartment (Mueller *et al*., 2004; Jeffery, 2005; Karniely and Pines, 2005).

The most surprising observation was that a subclass of proteins, which are known to be residents of internal mitochondrial compartments, i.e., matrix, inner membrane and intermembrane space, were found in the purified outer membranes and in a mitochondrial surface fraction generated by a mild protease treatment of mitochondria. Proteins of low and high abundance were about equally present in this subclass. An integrated analysis, combining protein localization (this study) and mRNA targeting-data (Marc *et al*., 2002), revealed that most of these proteins were characterized by a high score in a genome-wide analysis for mRNA targeting to mitochondria. mRNA targeting to mitochondria depends on the 3′ untranslated region and involves a mechanism conserved from yeast to human (Margeot *et al*., 2002; Sylvestre *et al*., 2003). So far, however, mRNA targeting has received little attention in the protein import field because mitochondrial protein import has been generally assumed to occur via posttranslational protein targeting without a specific function for mRNA targeting (Neupert, 1997; Truscott *et al*., 2003). The systematic enrichment of precursor proteins with a high mRNA targeting value MLR in the outer membrane proteome provides a first and extensive demonstration of the significance of mRNA targeting on the protein level. This conclusion was independently confirmed by a gradient separation of mitochondrial membrane vesicles, directly demonstrating the presence of the precursor form of a matrix-targeted protein in the outer membrane.

According to the endosymbiont hypothesis, mitochondrial proteins are derived from two different sources, the prokaryotic ancestor of mitochondria and the eukaryotic host cell (Karlberg *et al*., 2000; Marcotte *et al*., 2000). Marc *et al*. (2002) demonstrated that the prokaryote-derived and the eukaryote-derived mitochondrial proteins are significantly different in their MLR values such that the prokaryote-derived proteins typically possess high MLR values, i.e., their mRNAs are targeted to mitochondria (Karlberg and Andersson, 2003). Proteins in the outer membrane proteome and the mitochondrial surface fraction with high MLR values were highly enriched in this conserved subclass of proteins that are residents of internal mitochondrial compartments. The majority of resident outer membrane proteins do not possess prokaryotic homologues but are derived from the eukaryotic host cell (Pfanner *et al*., 2004), and indeed those proteins mostly display MLR values of low or middle magnitudes. These findings also shed new light on the ongoing discussion of a possible coupling of translation to protein translocation into mitochondria (Kellems *et al*., 1975; Verner, 1993; George *et al*., 2002). Indeed, the proteins fumarase (Fum1) and malate dehydrogenase (Mdh1), for which an experimental relation of protein synthesis to protein translocation has been reported (Knox *et al*., 1998; Fünfschilling and Rospert, 1999; Karniely and Pines, 2005), possess high MLR values and are found in the outer membrane proteome/mitochondrial surface fraction.

The accumulation of the conserved subclass of proteins at the mitochondrial surface was significantly enhanced under nonfermentable growth conditions that require high mitochondrial activity. Thus, large amounts of mitochondrial precursor proteins are synthesized. Because the TIM23 presequence translocase of the inner membrane is four-times less abundant than the outer membrane TOM complex (Dekker *et al*., 1997), the presequence pathway into the matrix may be overloaded under these conditions, leading to an accumulation of preproteins. Moreover, many proteins of this conserved subclass of proteins are subunits of large mitochondrial complexes such as the respiratory chain complexes that also contain subunits encoded by mitochondrial DNA. Thus, assembly of these complexes depends on the concomitant availability of numerous proteins synthesized in two different locations (Margeot *et al*., 2005). Under nonfermentable conditions, the rapid mitochondrial growth requires efficient assembly of respiratory chain complexes. Accumulation of the conserved subclass of proteins at the outer membrane under steady-state conditions will ensure a continuous supply (pool) of these proteins to minimize the problem that the conserved nuclear-encoded proteins would be limiting in mitochondrial assembly processes.

In summary, the combination of high-purity cellular subfractionation, comprehensive proteomic analysis, and integrative analysis of protein localization with mRNA targeting and evolutionary relationships provides important new insights into mitochondrial dynamics and biogenesis. The mitochondrial outer membrane is not simply a static membrane that only contains resident proteins; it also accumulates a conserved subclass of mitochondrial precursor proteins.

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