Mitochondria play a crucial role in cellular homeostasis, which justifies the increasing interest in mapping the different components of these organelles. Here we have focused our study on the identification of proteins of the mitochondrial inner membrane (MIM). This membrane is of particular interest because, besides the well known components of the respiratory chain complexes, it contains several ion channels and many carrier proteins that certainly play a key role in mitochondrial function and, therefore, deserve to be identified at the molecular level. To achieve this goal we have used a novel approach combining the use of highly purified mouse liver mitochondrial inner membranes, extraction of membrane proteins with organic acid, and two-dimensional liquid chromatography coupled to tandem mass spectrometry. This procedure allowed us to identify 182 proteins that are involved in several biochemical processes, such as the electron transport machinery, the protein import machinery, protein synthesis, lipid metabolism, and ion or substrate transport. The full range of isoelectric point (3.9–12.5), molecular mass (6–527 kDa), and hydrophobicity values (up to 16 transmembrane predicted domains) were represented. In addition, of the 182 proteins found, 20 were unknown or had never previously been associated with the MIM. Overexpression of some of these proteins in mammalian cells confirmed their mitochondrial localization and resulted in severe remodeling of the mitochondrial network. This study provides the first proteome of the MIM and provides a basis for a more detailed study of the newly characterized proteins of this membrane.

Mitochondria are eukaryotic organelles that play a crucial role in several cellular processes, including energy production, β-oxidation of fatty acids, the urea cycle, and programmed cell death. These complex organelles are composed of an outer and inner membrane defining an intermembrane space in which key regulators of apoptosis have recently been localized (1) and a matrix where the mitochondrial genome is confined. In mammals, the mitochondrial genome is about 16,500 base pairs long and encodes the 12 and 16 S rRNA, 22 tRNAs, and 13 polypeptides, all of which encode essential components of the respiratory chain. The low complexity of the mitochondrial genome indicates that the vast majority of the mitochondrial proteins (estimated to be 1,000) (2) are encoded by the nuclear genome. The mitochondrial inner membrane is of particular interest because it contains proteins of the respiratory chain as well as many carrier proteins and ion channels that certainly play a key role in mitochondrial function and cell homeostasis. Over the past few years, a number of approaches, such as two-dimensional PAGE and more recently one-dimensional PAGE, have allowed partial characterization of the proteome of entire mitochondria from various species (3–6). However, our knowledge of the protein composition of the mitochondrial inner membrane is still largely incomplete. Because such information would be invaluable for our understanding of the mitochondrial function, we have mapped the proteome of this membrane.

Until recently, the identification of new mitochondrial proteins mostly involved solubilization of entire cells or isolated mitochondria followed by two-dimensional gel analysis. However, the two-dimensional gel approach suffers from several major drawbacks. For example, many membrane proteins cannot be identified because of precipitation in the first dimension. Similarly, highly basic proteins are difficult to focus in the first dimension and thus are underrepresented in two-dimensional gel maps. Furthermore, low molecular mass proteins cannot be readily detected using this approach. Recently, a new method based on a two-dimensional liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) was used for the analysis of the yeast proteome (7). Using this method, Washburn et al. (7) identified 1,454 proteins, of which 131 harbor three or more predicted transmembrane domains. Therefore, two-dimensional LC/MS/MS can be successfully used to identify transmembrane proteins. In this study we have used a similar approach to map the proteome of the mitochondrial inner membrane from mouse liver (Scheme 1) and identified 182 proteins, of which 20 are to date uncharacterized.

**EXPERIMENTAL PROCEDURES**

Isolation and Purification of Mitochondria—Four female mice were killed by exposure to CO₂. Livers were excised and rinsed twice with ice-cold MB buffer (Mannitol 210 mM, Sucrose 70 mM, Heps 10 mM, EDTA 1 mM, pH 7.5). The washed livers were finely minced and homogenized in 60 ml of MB buffer using a glass homogenizer. The resultant homogenate was centrifuged at 2,000 × g for 5 min at 4 °C. The 2,000 × g supernatant was centrifuged twice at 11,700 × g for 10 min. The supernatant was centrifuged at 105,000 × g for 1 h. The mitochondrial pellet was resuspended in 8 ml M10 buffer (Mannitol 210 mM, Sucrose 70 mM, Heps 10 mM, EDTA 1 mM, pH 7.5) and stored at −80 °C. The mitochondrial pellets were used directly for protein extraction or stored at −80 °C for up to 3 months.

**MATERIALS AND METHODS**

**Sample preparation**

Mitochondrial outer membrane proteins were extracted using an organic acid method (2) and partially purified using the ammonium sulphate precipitation method (2). The purified mitochondrial inner membrane proteins were prepared using the organic acid method (2). The mitochondria were homogenized in 55 ml of MB buffer using a glass homogenizer. The homogenate was centrifuged at 11,700 × g for 10 min at 4 °C. The 11,700 × g supernatant was centrifuged at 105,000 × g for 1 h at 4 °C. The supernatant was centrifuged at 105,000 × g for 1 h at 4 °C. The mitochondrial pellet was resuspended in 8 ml M10 buffer (Mannitol 210 mM, Sucrose 70 mM, Heps 10 mM, EDTA 1 mM, pH 7.5) and stored at −80 °C. The mitochondrial pellets were used directly for protein extraction or stored at −80 °C for up to 3 months.

**Protein extraction**

The pelleted mitochondrial proteins were resuspended in 5 ml of M10 buffer containing 0.1% Triton X-100 and 2 µg/ml leupeptin. The mitochondrial pellet was stirred at 4 °C for 1 h at 4 °C. The mitochondrial pellet was resuspended in 8 ml M10 buffer (Mannitol 210 mM, Sucrose 70 mM, Heps 10 mM, EDTA 1 mM, pH 7.5) and stored at −80 °C. The mitochondrial pellets were used directly for protein extraction or stored at −80 °C for up to 3 months.
A discontinuous Nycodenz gradient was resuspended in 6 ml, loaded on fully layering 30 ml of a 33% MB Nycodenz solution over 5 ml of a 36% continuous Nycodenz gradient. This gradient was prepared by careful layering of MB buffer from a stock of 36% Nycodenz in MB buffer. Crude mitochondrial fractions (8 ml) were layered on top of the discontinuous Nycodenz gradient and centrifuged at 141,000 × g for 30 min. The 22.5 and 9.5% Nycodenz solutions were stepwise 10 ml of 22.5% and 17 ml of 9.5% Nycodenz solution in (Sigma) gradient (8). This gradient was prepared by carefully layering Mitochondrial Inner Membrane (MIM) (lane 1), mitoplasts (M, lanes 2 and 3), and mitochondrial inner membrane (MIM, lanes 6 and 7), were analyzed by SDS-PAGE and Western blot using antibodies against markers of the mitochondria (prohibitin), the endoplasmic reticulum (calnexin), and peroxisomes (catalase). B. samples of mitochondria after two consecutive Nycodenz gradients (M, lane 1), mitoplasts (M, lane 2), and mitochondrial inner membrane (MIM, lane 3) corresponding to 5 µg of total protein were analyzed by SDS-PAGE and Western blot using antibodies against markers of the MIM (prohibitin, adenine nucleotide translocator, ANT; cytochrome c oxidase subunit IV, COXIV), the matrix (mitochondrial heat shock protein 70, mHSP70), and the mitochondrial outer membrane (Bcl-XL).

min. The mitochondria-enriched pellet was gently homogenized in 50 ml of MB buffer in order to be purified on a discontinuous Nycodenz (Sigma) gradient (8). This gradient was prepared by carefully layering stepwise 10 ml of 22.5% and 17 ml of 9.5% Nycodenz solution in centrifuge tubes (35 ml). The 22.5 and 9.5% Nycodenz solutions were diluted in MB buffer from a stock of 36% Nycodenz in MB buffer. Crude mitochondrial fractions (8 ml) were layered on top of the discontinuous Nycodenz gradient and centrifuged at 141,000 × g for 1 h at 4 °C. The pellet obtained was resuspended in 20 ml of MB buffer and further centrifuged twice at 11,700 × g for 5 min before further purification on a continuous Nycodenz gradient. This gradient was prepared by carefully layering 30 ml of a 33% MB Nycodenz solution over 5 ml of a 36% MB Nycodenz solution. The mitochondrial pellet obtained after the discontinuous Nycodenz gradient was resuspended in 6 ml, loaded on six continuous gradients, and centrifuged at 39,000 × g for 1.5 h at 4 °C. The major band at the interface of the 36 and 33% Nycodenz solutions was collected, diluted in 3 volumes of MB buffer, and centrifuged twice at 11,700 × g for 10 min. The pellet obtained was resuspended in 2 ml of MB buffer, and the protein concentration was assessed using a BioRad Bradford assay.

Purification of Mitochondrial Inner Membrane (MIM)—Mitochondria were re-suspended in H2O at a concentration of 5 mg/ml and stirred for 20 min on ice. The mixture was homogenized 20 times with a glass homogenizer and centrifuged twice at 12,000 × g for 5 min. The mitoplasts obtained were treated with Na2CO3 0.1 M, pH 11.5, at a final concentration of 0.5 mg/ml for 20 min on ice. The mitochondrial inner membrane was pelleted at 100,000 × g for 30 min.

Solubilization and Digestion of MIM—500 µg of MIM were solubilized in 100 µl of 90% formic acid. After adjusting the pH to 8.5 with ammonium carbonate, the sample was sequentially treated with 8 M urea, reduced by adding diethiothreitol (1 mM), and carboxymethylated in 10 mM iodoacetamide. The solution was diluted to 2 M urea with 100 mM ammonium bicarbonate, pH 8.5, and finally digested with trypsin (1/20) for 24 h. The tryptic digest was centrifuged at 100,000 × g for 30 min at 4 °C. A solid phase extraction with SPEC-PLUS C18 columns (Ansys) was carried out to concentrate the supernatant and buffer exchange the mixtures into acetoni trile, 0.1% acetic acid. The sample was then dried.

Strong Cation Exchange—500 µg of peptide digest was loaded onto a strong cation exchange Polysulfoethyl A column (2.1 mm × 20 cm) (PolyLC Inc.) 0.2 ml/min. The peptides were eluted on a salt step gradient: 0, 30, 50, 70, and 100% B. Mobile phase buffers were A (10 mM KH2PO4, 25% acetonitrile, 500 mM KCl). 23 fractions were collected and concentrated using SPEC PLUS C18 columns as described above.

Electrospray LC/MS/MS—Each fraction was further analyzed by LC/MS/MS. All data were acquired using a Q-ToF Ultima mass spectrometer, hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, fitted with a nanoflow electrospray source, coupled on line with an analytical CapLC system (Waters, Manchester, UK). The HPLC was configured with a preconcentration column to allow large volume sample injection and a nanoscale column and a nanobore column. The trapping column was packed with 5 µm Symmetry C18 stationary phase (300 µm inner diameter × 5 mm), whereas the analytical column was a 150-mm × 75-µm column packed with PepMap C18 material. The reverse phase column was eluted into the mass spectrometer with a linear gradient from 5 to 100% acetonitrile, 0.1% formic acid at 200 nanoliters/min.

Analysis of MS/MS data—To analyze the 21,000 spectra in a semi-automated fashion, a set of tools has been developed around the Mascot program (9). The approach was to incorporate rules derived from a set of manually curated MS/MS spectra in the Mascot search and to use this set of spectra to train our algorithm similarly to Keller’s SEQUEST approach (10). Receiver-to-operator plots were used to define ranges of accuracy and coverage. Using the training data set without these rules, only ~40% of the training spectra were assigned. However, using these
Mitochondrial Inner Membrane Proteins

**TABLE I**
Uncharacterized proteins

| Ensembl name  | TM segments | NB peptides | Swiss-Prot | Protein name |
|---------------|-------------|-------------|------------|--------------|
| ENSMUS P0000035804  | 1          | 89          | Q9CW42     | Unknown (39270 Da), (pI = 7.82) |
| ENSMUS P0000027930  | 1          | 49          | Q922Q1     | Unknown (protein for MGC-6272) (38194 Da), (pI = 9.39) |
| ENSMUS P0000024330  | 1          | 25          | Q9ER48     | Up-regulated during skeletal muscle growth 5 (6381 Da), (pI = 10.32) |
| ENSMUS P0000030903  | 1          | 19          | Q92511     | TOB3 (66742 Da), (pI = 9.32) |
| ENSMUS P0000049338  | 0          | 18          | Q5S125     | NIPSNAP1 protein (33363 Da), (pI = 9.85) |
| ENSMUS P0000009431  | 1          | 17          | Q8Z210     | Leucine zipper EF-hand containing transmembrane protein 1 (82988 Da), (pI = 6.47) |
| ENSMUS P0000030169  | 0          | 17          | Q6DCG8     | Stomat-like protein 2 (SLP-2) (38385 Da), (pI = 9.25) |
| ENSMUS P0000023071  | 1          | 8           | Q9YS12     | Protein CGI (51905 Da), (pI = 8.555) |
| ENSMUS P0000031776  | 0          | 7           | Q9DF91     | RIKEN CDNA L69 (26534 Da), (pI = 8.57) |
| ENSMUS P0000026596  | 3          | 7           | Q9CE24     | 9430063G14Rik Protein (29260 Da), (pI = 9.8) |
| ENSMUS P0000045654  | 2          | 5           | Q9JLG5     | Brain protein 44-like protein (18528 Da), (pI = 10.02) |
| ENSMUS P0000032634  | 16         | 3           | Q8473      | HERC2 protein (527369 Da), (pI = 6.02) |
| ENSMUS P0000027683  | 0          | 2           | Q81267     | GAP2 (45597 Da), (pI = 5.35) |
| ENSMUS P00000042469 | 4          | 1           | Q9JJ2O     | Putative N-acetyl transferase catalytic domain (25010 Da), (pI = 9.61) |
| ENSMUS P0000028793  | 1          | 1           | Q6TC75     | Involved in anti-apoptotic signaling (30967 Da), (pI = 8.48) |
| ENSMUS P0000018913  | 3          | 1           | Q9DSW3     | 1810027010Rik protein (11655 Da), (pI = 9.19) |
| ENSMUS P0000031039  | 2          | 1           | Q9DSW7     | 1810027120Rik protein (16925 Da), (pI = 9.95) |
| ENSMUS P0000024973  | 0          | 1           | Q9JBB2     | Ambiguous (25172 Da), (pI = 7.8) |
| ENSMUS P0000043028  | 3          | 1           | RAC35287   | Hypothetical DNAJ N-terminal domain containing protein (63336 Da), (pI = 8.03) |
| ENSMUS P0000002403  | 3          | 1           | Q9D145     | 1110029G07Rik protein (34005 Da), (pI = 8.46) |

* Accession number in Ensembl mouse genome.
* Number of transmembrane segments predicted by TMpred.
* Number of peptides identified by LC/MS/MS.
* Accession number in Swiss-Prot.

rules ~80–85% of the training’s spectra were correctly interpreted. Still, 15–25% of the MS/MS spectra were either unidentified or incorrectly assigned, mainly because of very low signal to noise ratio, chemical and peptide mixtures. The final assessment of each protein identified was performed manually.

**DNA Cloning**—All the constructs in this study were generated by standard PCR cloning procedures using either a mouse liver Marathon-ready cDNA library (Clontech) or human colon, follicular dendritic, and myeloma cDNA libraries (generously provided by Dr. Garry Buell, Serono Pharmaceutical Research Institute). The amplified sequences were N- or C-terminal fused to FLAG (DYKDDDDK) or HA (YPYDVPDYA) tags by PCR and cloned into the pCI mammalian expression vector (Promega). All the constructs were sequenced to verify the fidelity of the cDNA sequences obtained.

**Immunocytochemistry**—HeLa cells were plated on glass coverslips in 12-well plates (Nunc) at a density of 0.5 × 10⁶ per well. 12–16 h after plating, the cells were transfected with the relevant constructs using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. 48 h after transfection the cells were fixed in 4% paraformaldehyde/PBS for 20 min at room temperature (RT), permeabilized in 0.1% saponin/PBS for 15 min at RT, and blocked for 1 h in 0.1% saponin/PBS with 5% goat serum. The coverslips were incubated for 1 h with primary antibodies in 0.1% saponin/PBS with 5% goat serum: polyclonal anti-FLAG (DYKDDDDK) (1:1000; Sigma), monoclonal anti-HA (YPYDVPDYA) (1:100; Babco), and monoclonal anti-mitochondrial HSP70 (1:50; BIOSOURCE). The cells were washed three times in 0.1% saponin/PBS with 5% goat serum, and the immune complexes were revealed by incubating the coverslips with fluorescent isothiocyanate (FITC)-conjugated anti-mouse (1:100; Vector labs) or FITC-conjugated anti-rabbit (1:100; Vector labs) and Texas Red-conjugated anti-mouse (1:100; Vector labs) secondary antibodies. Finally, the cells were washed three times in 0.1% saponin/PBS and mounted using Vectashield H-1000 fluorescent mounting medium (Vector labs). Transfected cells were viewed on an inverted microscope (Axiovert 135TV; Zeiss) with ×100 objective, and images were captured using a CCD camera (photometric CE200A) with IP Lab software. The resulting images were processed using Adobe Photoshop 6.

**Cell Viability Assay**—HeLa cells were transfected as above with 0.5 μg of target DNA together with 0.5 μg of pCI-Luc, in the presence or absence of zVAD (50 μM). All transfections were performed in 105 per well. 12 h after transfection, cells were washed once in PBS and lysed in 900 μl of luciferase assay buffer (Promega) for 20 min at room temperature. 10 μl of the extract was mixed with 50 μl of luciferase assay reagent II (Promega) and immediately measured using a Turner TD-20e luminometer.

**Mitochondrial Inner Membrane Proteins**—In general, membrane-inserted proteins display one or several transmembrane domains separated by extramembrane loops of variable lengths. In many cases positively charged amino acids such as lysines and arginines are found at the junction between the transmembrane segments and the loops (11) and represent potential trypsin digestion sites. To generate as many peptides as possible from membrane-inserted proteins and thereby maximize the chances of their identification by MS, the MIMs were solubilized with formic acid to allow peptide mixtures. The final assessment of each protein identified was performed manually. The goal of the alkali treatment was to eliminate peripherally associated membrane proteins and proteins from the matrix. The mitochondrial and MIM purifications were assessed by SDS-PAGE and immunoblotting with antibodies against bona fide markers of the different mitochondrial compartments (matrix, mHap70; mitochondrial outer membrane, Bcl-XL; inner mitochondrial membrane, COX IV, Prohibitin, ANT). Enrichment of the MIM was monitored by the increase in the ratio of levels of the MIM markers/mg of protein. The purest MIM preparations obtained had a ratio of Prohibitin/mg of protein between 10–20-fold higher than that of purified intact mitochondria (Fig. 1A and data not shown). A similar enrichment was obtained with ANT and COXIV (Fig. 1B).

**RESULTS AND DISCUSSION**—Mitochondria from mouse liver have been purified on a Nycodenz gradient, and mitoplasts were obtained following mechanical disruption of the outer mitochondrial membrane. An alkali treatment of the mitoplasts resulted in highly enriched MIM.

**Identification of Mitochondrial Inner Membrane Proteins**—The goal of the alkali treatment was to eliminate peripherally associated membrane proteins and thereby maximize the chances of their identification by MS. The MIMs were solubilized with formic acid to allow access of transmembrane segments to trypsin as well as to the extramembrane loops, as previously described (7). To reduce the complexity of the sample, the peptides resulting from the above treatment were separated on a strong cation exchange
Each fraction collected from the SCX column was then analyzed by nanoscale liquid chromatography coupled to LC/MS/MS. 21,000 MS/MS spectra were obtained, and a first analysis was performed using Mascot. Because of the large amount of data generated, it was necessary to develop a software tool to automate the analysis of the spectra (see “Experimental Procedures”). The list of proteins presented in Supplementary Table I was obtained using this software and confirmed by manual analysis. In total, 182 proteins were identified, of which 45% harbor 2–16 transmembrane domains (as predicted using TMpred), indicating that the procedure used is appropriate to identify membrane-inserted proteins. A few proteins (19%) do not display a transmembrane domain and are therefore unlikely to be inserted in the membrane. These proteins are probably peripherally attached to the membrane and were resistant to the alkali treatment. Our approach allowed the characterization of proteins with a wide pI range (3.9–12.5); in fact, 50% of the proteins analyzed display a pI over 9 or below 4. It also allowed the identification of 11 proteins with a mass lower than 10 kDa and 8 larger than 100 kDa (Supplementary Fig. 1). The number of peptides leading to the identification of each protein varied from one, for proteins of low abundance, to several hundred for abundant proteins. For example, 840 peptides were found for ANT and 504 for F1F0ATPase, which is consistent with these two proteins being the most abundant proteins of the MIM (25). However, in addition to the abundance criteria, the number of tryptic peptides generated for a given protein may be modulated by factors such as the size, the amino acid composition, and the accessibility to trypsin. Therefore, the number of peptides contributing to the identification of a protein can only be taken as a semi-quantitative measure of its abundance in the sample. 3.5% of the proteins listed in Supplementary Table I are known to reside outside of mitochondria. These proteins could either be minor contaminants or could have a mitochondrial residence.
so far ignored. In addition, the MIM preparation appeared to be slightly (5%) contaminated by proteins known to localize to the mitochondrial outer membrane, including voltage-dependent anion-selective channel (VDAC) and carnitine palmitoyltransferase I. These proteins are known to be present at contact sites between the inner and the outer mitochondrial membranes (12, 13). Their presence indicates that segments of the outer mitochondrial membrane forming these contact sites were purified with the MIM. In addition, a number of mitochondrial matrix proteins (14% of the total number of proteins identified), such as the carbamoyl phosphate synthase or the malate dehydrogenase, were found in their precursor form. This suggests the N-terminal targeting signals of these proteins had not yet been processed by the mitochondrial matrix peptidase and thus might not have yet been released from the translocase of outer membrane (TOM/translocase of inner membrane (TIM) protein translocation complex. Alternatively, these matrix proteins that display a putative transmembrane domain could actually be integral membrane proteins of the inner membrane that have substantial domains on the matrix side of the membrane. In summary, the approach used in this study successfully led to the identification of proteins with widely varied molecular mass ranges, pI values, and hydrophobicities. Moreover, the list in Supplementary Table I shows that there are molecular mass ranges, pI values, and hydrophobicities. More-fully led to the identification of proteins with widely varied

Novel Mitochondrial Inner Membrane Proteins—Of a total of 182 proteins identified in this study, 15 are unknown and 5 had previously been described but had never been localized to the mitochondria (Table I). To determine whether these uncharacterized proteins are truly associated with the mitochondria, several software programs designed to predict subcellular localization were used (MITOPROT, Predotar). However, the results of these predictions were often divergent, depending on which software was used. Therefore, some of the uncharacterized proteins were fused to HA or FLAG epitope tags and their subcellular localization was assessed after overexpression in HeLa cells. As shown in Fig. 2, all fusion proteins showed a punctate or filamentous pattern after immunostaining with an anti-HA or anti-FLAG antibody. In each case the anti-HA or anti-FLAG staining co-localized with the mitochondrial network visualized by co-expression with a mitochondrially targeted YFP fusion protein or by co-immunostaining with an antibody against the mitochondrial form of Hsp70.

During the course of these experiments, it was observed that overexpression of some of these proteins was followed by a drop in the number of healthy transfected cells. To quantify the toxic effects of the expression of some of these proteins, HeLa cells were co-transfected with a luciferase reporter construct and the activity of luciferase, 48 h after transfection, was taken as an index for cell viability (see "Experimental Procedures"). As shown in Fig. 3, expression of all the fusion proteins, except for the HA-tagged stomatin-like protein 2 (SLP-2), resulted in a distinct drop in luciferase activity as compared with the vector alone, suggesting that overexpression of these proteins was toxic for these cells. To test whether apoptosis was induced by overexpression of these proteins, zVAD, a pan-caspase peptide inhibitor, was added to the culture medium following transfection of the different constructs. Addition of zVAD resulted in a significant increase in luciferase activity, suggesting that overexpression of these proteins (except SLP-2-HA) induces caspase-dependent apoptosis.

As shown in Table I, the majority of 20 uncharacterized proteins display one or more transmembrane domains, their molecular mass ranges from 6 to 527 kDa, and some appeared to be abundant (89 and 49 peptides were obtained for Q9CW42 and Q922Q1, respectively). Q922Q1 cDNA, encoding one of these abundant novel proteins, was cloned in a mammalian expression vector fused to a FLAG tag. This protein harbors a MOSC sequence predicted to be a sulfur-carrier domain that delivers sulfur for the formation of diverse sulfur-metal clusters (14). As shown in Fig. 2, upon overexpression in HeLa cells, the FLAG-Q922Q1 fusion protein co-localized with mitochondrial Hsp70, thus confirming its association with the mitochondrial network. Interestingly, overexpression of FLAG-Q922Q1 induced the collapse of the mitochondrial tubular network and its aggregation around the nucleus.

Among the uncharacterized proteins listed in Table I, Q92511 (also called TOB3) was examined in more detail. TOB3 displays a single predicted transmembrane segment and an
AAA-ATPase domain that can also be found in other mitochondrial inner membrane proteases such as AFG3L (15), which was also identified in our study. When the localization of TOB3 fused to a FLAG tag was assessed using the same approach as presented above, it was found to be clearly mitochondrial (Fig. 2). In addition, prolonged expression of the FLAG-TOB3 protein resulted in fragmentation of the mitochondrial network (Fig. 4) and cell death as mentioned above (Fig. 3). Interestingly, sequence analysis shows that the closest homologue of TOB3 in yeast is YME1, a mitochondrial AAA protease (Table II). YME1 plays an essential role in the degradation of unassembled or misassembled subunits of cytochrome c oxidase (16). YME1 gene disruption led to several dysfunctions, including a reduced activity of the respiration chain complexes, slow growth, and lethality when the mutation is expressed in a p+ mitochondrial background (17). Moreover, the inactivation of the homologue of TOB3 in Caenorhabditis elegans is lethal (Table II) (18). Conversely, the data presented in Fig. 3 indicate that the overexpression of TOB3 in mammalian cells led to a drop in cell survival. Thus, it seems that either the lack or the excess of TOB3 at the mitochondria might upset the homeostasis of this organelle and thus lead to cell death.

Another uncharacterized protein, Q9JJB2 or SLP-2, was investigated further. No transmembrane domain is predicted for this protein. To confirm that SLP-2 was not a cytoplasmic contaminant of our MIM preparation, the protein fused to an HA tag was ectopically expressed in HeLa cells. As shown in Fig. 2, SLP-2-HA co-localizes with mitochondrially targeted YFP fusion protein. Our results are also supported by a recent study (6) of the human mitochondrial proteome showing that YFP fusion protein. Our results are also supported by a recent study (6) of the human mitochondrial proteome showing that hydrophobic domains that is commonly found in the stomatin gene family. Stomatins are integral membrane proteins that play a major role in the regulation of ion channels by interacting with proteins of the cytoskeleton. In C. elegans, a stomatin homologue (MEC-2) is required for sensory mechanoreception and the gating of an oligomeric sodium channel (21). UNC-24, a protein most similar to a human stomatin homologue (SLP-1), is also required for locomotor response to volatile anesthetics in C. elegans (22). Thus, SLP-2 might be involved in regulating ion channels of the MIM and could represent an interesting tool to identify these ion channels.

CONCLUSION

Using a preparation of highly purified MIM, we have identified 20 unknown mitochondrial proteins. Although our approach represents major progress compared with previous studies, the proteome of the mitochondrial inner membrane is still incomplete because our studies did not allow identification of ion channels such as calcium or ATP-dependent potassium channels that are supposed to be present in this membrane (23, 24). Nevertheless, novel proteins have been identified, the function of which may be crucial for mitochondrial function and mammalian cell homeostasis as predicted by the severe phenotypes resulting from mutations of their homologues in yeast and in C. elegans. Our discovery that these genes encode mitochondrial inner membrane proteins is a first step toward a better understanding of the mechanism of action of these crucial proteins.

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