A Systematic Characterization of Mitochondrial Proteome from Human T Leukemia Cells*

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Global understanding of tissue-specific differences in mitochondrial signal transduction requires comprehensive mitochondrial protein identification from multiple cell and tissue types. Here, we explore the feasibility and efficiency of protein identification using the one-dimensional gel electrophoresis in combination with the nano liquid chromatography tandem mass spectrometry (GeLC-MS/MS). The use of only 40 μg of purified mitochondrial proteins and data analysis using stringent scoring criteria and the molecular mass validation of the gel slices enables the identification of 227 known mitochondrial proteins (membrane and soluble) and 453 additional proteins likely to be associated with mitochondria. Replicate analyses of 60 μg of mitochondrial proteins on the faster scanning LTQ mass spectrometer validate all the previously identified proteins and most of the single hit proteins except the 81 single hit proteins. Among the identified proteins, 466 proteins are known to functionally participate in various processes such as respiration, tricarboxylic acid cycle (TCA cycle), amino acid and nucleotide metabolism, glycolysis, protection against oxidative stress, mitochondrial assembly, molecular transport, protein biosynthesis, cell cycle control, and many known cellular processes. The distribution of identified proteins in terms of size, pI, and hydrophobicity reveal that the present analytical strategy is largely unbiased and very efficient. Thus, we conclude that this approach is suitable for characterizing subcellular proteomes form multiple cells and tissues. Molecular & Cellular Proteomics 4: 169–181, 2005.

Mitochondria are one of the most complex and important organelles found in eukaryotic cells. In addition to their central role in energy metabolism, mitochondria are involved in many cellular processes and mitochondrial dysfunctions have been associated with apoptosis, aging, and a number of pathological conditions, including Parkinson’s, diabetes mellitus, Alzheimer’s, and cardiovascular diseases (1, 2). The fundamental role of mitochondria in cell life and death has driven experimental efforts to define mitochondrial proteome and to discover new molecular target for drug development and therapeutic intervention. In mammals, the mitochondrial genome is approximately 16,500 nucleotides long and encodes the 12 and 16S 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 vast majority of the mitochondrial proteins (estimated to be 1,500) are encoded by nuclear genome (1–3). So far, the largest proteomic study of purified human heart mitochondria was performed by Taylor et al., leading to the identification of 615 mitochondrial and mitochondria-associated proteins with a coverage of ~45% of the predicted human mitochondrial proteins (4, 5). However, to achieve this number of protein identifications, 701 LC-MS/MS runs were carried out with a starting material of 40 mg of mitochondrial proteins. MitoProteome database, a recently developed publicly accessible database, lists 784 mitochondrial genes and proteins for human, well short of the predicted human mitochondrial proteins (6). Traditionally, two-dimensional (2D) PAGE has been the method of choice for the characterization of a complex protein mixture prior to MS analysis followed by enzymatic digestion of the separated protein spots (7, 8). In 2D-PAGE, due to the separation of protein into multiple gel spots, subsequent digestion, extraction, and LC-MS/MS analysis of each spot are tedious and time consuming. Moreover, despite the recent advances, this approach is biased against membrane-associated proteins, low-abundance proteins, or proteins with extremes in isoelectric point or molecular mass (9, 10). To overcome the limitations of 2D-PAGE, approaches which combine one or several chromatographic separations with tandem mass spectrometry have been developed (11–13). One promising method, termed multidimensional capillary-scale LC-ESI-MS/MS protein identification technology (MudPIT) and pioneered by Yates and colleagues, permits shotgun sequencing of large numbers of proteins present in complex mixtures (11–13). MudPIT has been applied successfully to several model organisms, leading to identification of 1,484 proteins in yeast, 2,363 proteins in rice, and most recently 2,415 proteins in plasmodium (12, 14, 15), thus suggesting that MudPIT is much more powerful for the global characterization of proteins from cells and tissues compared with the

*The abbreviations used are: 2D, two dimensional; 1D, one dimensional; GeLC-MS/MS, 1D PAGE protein separation followed by nano-capillary LC-MS/MS; TMD, transmembrane domain; hnRNP, heterogeneous nuclear ribonucleoprotein; MudPIT, multidimensional capillary-scale LC-ESI-MS/MS protein identification technology; LDH, lactate dehydrogenase; HFBA, heptafurobutyric acid; PCNA, proliferating cell nuclear antigen; TCA, tricarboxylic acid.
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2D-PAGE. However, successful analysis using the MudPIT strategy requires proficiency and sophistication in multidimensional chromatography to prevent sample loss associated with each of the chromatography steps.

An alternative approach that is simple and easily performed by many in the field of proteomics with very little expertise is one-dimensional (1D)-SDS-PAGE protein separation and nano-LC-MS/MS. Recently, this method has been successfully employed for profiling proteome of mitochondria and human cell lines (4, 16, 17). In this study, we have used a similar approach to create a reference proteome database of mitochondria from human Jurkat T leukemia cells. This cell line has been extensively utilized to study apoptosis, and all of these cells synchronously undergo apoptosis when the Fas receptor is engaged (1–3, 18). Furthermore, identification of the mitochondrial protein makeup of this cell line may provide additional biological insights of leukemic transformation.

From the sucrose gradient-purified mitochondria, we have identified 680 mitochondrial and mitochondria-associated proteins, of which 227 are known mitochondrial proteins. Thus, this study provides the first large-scale analysis of human leukemia cells and allows further comparative analysis of tissue-specific protein expression in the mitochondria.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-cytochrome c antibody (7H8.2 c12, 6H2.B4; BD Pharmigen, San Diego, CA); cytosolic marker antilactate dehydrogenase (LDH; Sigma, St. Louis, MO); nuclear marker anti-PCNA (clone PC10; Oncogene Research Products, San Diego, CA); anti-Fas (Molecular Probes, Eugene, OR). All other reagents were from Sigma.

Cell Culture—The human T leukemia cells (Jurkat A3) were obtained from the American Type Culture Collection (Bethesda, MD). Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 25 mM HEPES, and antibiotics in a humidified incubator with 5% CO2 in air at 37 °C. The cells were inactivated FBS, 2 mML -glutamine, 25 mM HEPES, and antibiotics in a humidified incubator with 5% CO2 in air at 37 °C. The cells were grown to a maximum density of 0.5–0.8 × 10^6/ml and split at a ratio of 1:10.

Subcellular Fractionation and Western Blotting—Mitochondria were isolated as described previously with minor modifications as outlined below (19). Jurkat A3 cells were collected by centrifugation at 400 × g for 10 min at 4 °C. The cell pellets were washed twice with ice-cold PBS (pH 7.4) and resuspended with 10 volumes of isolation buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.25 M sucrose, and a mixture of protease inhibitors). After 10-min incubation on ice, the cells were homogenized in a glass Dounce homogenizer until 75% of the cells became trypan blue-positive. The homogenates were centrifuged twice at 650 × g for 10 min at 4 °C to remove nuclei and unbroken cells. The postnuclear supernatants were centrifuged at 12,500 × g for 25 min at 4 °C, and the pellets were saved as the heavy membrane fraction (designated HM). The supernatants of the 12,500 × g spin were further centrifuged at 100,000 × g for 1 h at 4 °C, and the resulting supernatants (designated cytosolic; S-100) and pellet (designated light membrane; LM) were frozen as aliquots at −80 °C for subsequent experiments. The heavy membrane fraction was resuspended carefully in the isolation buffer and centrifuged again at 12,500 × g for 25 min. The heavy membrane fraction was then resuspended in isotonic sucrose buffer (0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4), layered on a 1.0/1.5 M discontinuous sucrose gradient, and centrifuged at 60,000 × g for 20 min at 4 °C. The mitochondria were collected from the phase between the 1.0 and 1.5 M sucrose, diluted in the isolation buffer, and centrifuged again at 15,000 × g for 20 min to pellet mitochondria. Purified mitochondrial pellets were washed with isolation buffer and then preserved at −80 °C until further analysis.

Purified mitochondrial fraction and HM fraction were solubilized in lysis buffer (1% n-dodecyl-β-D-maltoside, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and a mixture of protease inhibitors) for 1 h on ice and centrifuged at 15,000 × g for 5 min. The supernatant was collected, and protein concentration was determined by a Micro-BCA protein concentration determination kit (Pierce, Rockford, IL). For Western blotting, equal amount of various subcellular fractions were loaded in each lane of a 10% NuPAGE gel (Invitrogen, San Diego, CA). After gel electrophoresis and protein transfer, the membranes were probed with various primary and corresponding secondary antibodies against marker proteins from different cellular compartments. Immunoreactivity was detected with an ECL method (PerkinElmer, Boston, MA).

In-gel Digestion with Trypsin—A total mitochondrial protein from 0.75 × 10^7 cells (40 μg) was loaded on 10% NuPAGE gel (Invitrogen) and run at 20 mA for 30 min, then 30 mA for 2 h. The gel was stained with Coomassie blue R-250 (50% methanol, 1% acetic acid, 0.1% R-250) for 30 min and destained overnight in a solution containing 5% methanol, 7% acetic acid. After imaging, the area from the top to the bottom of each lane of the Coomassie-stained gel was cut at 2-mm intervals (some slices were wider because of the absence of any prominent band at those positions). Each gel slice was cut into small pieces (~1-mm cubes) and transferred to 500-μl microcentrifuge tubes. The gel pieces were washed with 200 μl of 50 mM NH4HCO3 for 45 min at room temperature. The supernatant was removed and then 200 μl of neutralization buffer (50 mM NH4HCO3 in 50% CH3CN) was added for 20 min at room temperature. The gel pieces were completely neutralized and destained by repeated washes with NH4HCO3 and NH4HCO3/CH3CN if necessary. The destained gel pieces were dehydrated with 100% CH3CN and dried in a vacuum concentrator (CentriVap, Labconco Corporation, Kansas City, MO). Gel pieces were rehydrated with 15–20 μl of trypsin solution (25 ng/μl in 100 mM NH4HCO3) on ice for 45 min. In-gel digestion was performed at 37 °C for 18–20 h. The resulting peptides were extracted according to the protocol of Shevchenko et al. (20). Extracted peptides were dried in Centriviap, redissolved in solvent A [5% ACN, 0.4% acetic acid, and 0.005% heptfluorobutyric acid (HFBA)] and stored at −20 °C until mass spectrometric analysis was performed. For the two additional replicate analyses, 60 μg of mitochondrial proteins were analyzed on a faster scanning Ion trap mass spectrometer (Finnigan LTQ; Thermo Finnigan, Palo Alto, CA).

Nano-LC-MS/MS Analysis—The digested mitochondrial proteins were sequenced using a high-throughput tandem mass spectrometer (LCQ-DECA ion trap; Thermo Finnigan) equipped with an in-house built nano-electrospray device. Samples were directly loaded onto a 10-cm × 75-μm capillary reverse-phase column packed in-house (Magic C18; Michrom BioResources, Auburn, CA) by means of a helium pressure cell. The column was previously equilibrated with solvent A [5% ACN, 0.4% acetic acid, and 0.005% HFBA]. Peptides were eluted with a linear gradient from 100% solvent A to 80% solvent B (95% ACN, 0.4% acetic acid, and 0.005% HFBA) for 85 min at a flow rate of ~240 nl/min. Peptides were eluted directly into the LCQ-DECA ESI Ion trap mass spectrometer capable of data-dependent acquisition. Each full MS scan was followed by two MS/MS scans of the two most intense peaks in the full MS spectrum with dynamic exclusion enabled to allow detection of less-abundant peptide ions. Mass spectrometric scan events and HPLC solvent gradients were controlled by the Xcalibur software (Thermo Finnigan). For two addi-
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FIG. 1. Purification and characterization of Jurkat T cell mitochondria. A, equal amounts of proteins (15 μg) were loaded onto a 10% SDS-PAGE and analyzed by Western blotting with indicated antibodies against marker proteins from mitochondria, cytosol, or nucleus. Antibodies against LDH and PCNA were used as markers for cytosolic and nuclear/cytosolic fractions, respectively. Antibodies directed against F0F1 ATP synthase subunit α and cytochrome c were used as markers for mitochondrial fraction. B, electron micrograph of purified mitochondrial fraction is shown indicating the longitudinal and cross-sections of mitochondria. Bar, 1 μm.

RESULTS AND DISCUSSION

Characterization of Isolated Jurkat Mitochondria—The quality of a subcellular proteome, such as that of mitochondria, is largely dependent on its purity. Thus, we optimized the purification procedure that allowed the isolation of high-purity mitochondria from Jurkat T leukemia cells. This procedure involved three differential centrifugations followed by the sucrose density gradient centrifugation using three sucrose densities (19). Before the sucrose gradient centrifugation, crude mitochondrial pellet was resuspended in mitochondrial isolation buffer and pelleted at 4,000 × g for further enrichment of mitochondria. The purity of mitochondria was subsequently assessed by Western blotting of known proteins from mitochondria (respiratory proton pump F1α subunit and cytochrome c), one abundant cytosolic marker protein (LDH), and a nuclear-cytosolic marker protein (proliferating cell nuclear antigen (PCNA)). As shown in Fig. 1A, F1α and cytochrome c proteins were specifically detected in the purified mitochondrial fraction, and this fraction lacked any detectable contamination of abundant cytosolic and nuclear proteins such as the LDH and PCNA. These results were further validated by electron microscopic analysis of purified mitochondrial fraction where most of the structures detected showed intact mitochondria with typical membrane architecture (Fig. 1B).

NANO-LC-MS/MS Analysis and Proteins Identification—To identify the proteome make-up of Jurkat T leukemia cell mitochondria, we first dissolved the purified mitochondria in a buffer optimized for solubilizing mitochondrial proteins as described in “Experimental Procedures” (4). The mitochondrial proteins were separated by 1D-NuPAGE gel (10%) and visualized by Coomassie staining. As shown in Fig. 2A, the complexity of isolated mitochondrial proteins range from ~8 to over 300 kDa in molecular mass, indicating that the purification procedure did not cause detectable protein degradation. Because our purpose was to identify mitochondrial and mitochondria-associated proteins without any bias, we analyzed all of the detectable proteins (22 gel slices) from the top to the bottom of the gel using the nano-LC-MS/MS method as described in “Experimental Procedures.” The MS/MS spectra obtained from 22 LC-MS/MS runs were searched against the human NCI nonredundant database using the SEQUEST algorithm running on a Linux cluster with 32 nodes (21). From the 22 LC-MS/MS runs, 32,298 MS/MS attempts were per-
FIG. 2. Proteomic characterization of purified Jurkat mitochondria. A, total mitochondrial lysate (40 μg) was separated by the use of 10% gel and visualized by Coomassie blue staining. Twenty-two gel slices were excised from this gel, subjected to in-gel trypsin digestion followed by the nano-capillary LC-MS/MS analysis. Molecular mass markers are shown on the left. Regions of the gel slices are shown on the right. Identified protein names and their theoretically calculated molecular masses from gel slice number 16 is listed on the right of the gel. B,
Most effective filtering criterion (Fig. 2) cut-offs revealed that the probability value of 0.85 was the significant loss of true protein identifications. Table I shows the results using different probability value cut-offs (Pcomp).

Total number of MS/MS attempts from validation experiments, experiment 2 and 3, are also shown.

Fig. 2 shows the percentage of proteins identified with varying numbers of peptides by the 1D GeLC-MS/MS strategy. Similar to most proteomics studies, about 18% of proteins in this analysis were identified from a single peptide (12, 13). Proteins identified by ≥2 peptides accounted for over 80% of the proteins in the mitochondrial fraction (Fig. 3). This study, with only 40–60 μg of mitochondrial proteins as the starting material, followed by simple LC-MS/MS analysis of 22 gel slices and two validation experiments, afforded the conclusive identification of 599 proteins. Thus, this simple technology seems to be the most effective way to identify a large number of proteins from different cell types. Furthermore, this is the first large-scale study where mitochondrial proteome from the Jurkat T leukemia cell line was carefully characterized, providing tissue-specific proteomic information for these cells.

**Physiochemical Characteristics of the Identified Proteins**—The identified proteins were then classified based on different physiochemical characteristics such as mass, pl, hydrophobicity (GRAVY value), and subcellular localization by the presence of TMD using the SOSUI algorithm (25). In this present work, more proteins with molecular mass above 100 kDa and below 10 kDa were observed than typical 2D gel separation. The smallest and the largest molecular mass obtained are 8.6 and 469.2 kDa respectively. Among the 680 identified proteins, 514 (75.6%) distribute among 10–80-kDa molecular mass intervals. Moreover, we found three proteins (0.5%) with mass less than 10 kDa and 112 (16.5%) with mass greater than 100 kDa, beyond the general 2D-PAGE separation limits (Fig. 4A). The domination of small-molecular-mass proteins (20–40 kDa) in mitochondrial fraction is consistent with the previous study of human heart mitochondrial proteome (4). Because of the limitation of 10% 1D-PAGE, we identified only mobility (Fig. 2A). When we examined whether multiple peptides from different gel slices resulted in the single protein identification, we found that except for the 82 proteins that were present in two adjacent gel slices, all of the remaining protein identifications were from a single gel slice (Fig. 2A and Supplemental Table IV).

Table I: Filtering criteria for protein identification using PeptideProphet software tool

<table>
<thead>
<tr>
<th>Sequest output files: 32,298</th>
<th>Pcomp ≥ 0.5</th>
<th>Pcomp ≥ 0.7</th>
<th>Pcomp ≥ 0.85</th>
<th>Pcomp ≥ 0.95</th>
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<tr>
<td>Total peptides</td>
<td>4,588</td>
<td>3,900</td>
<td>3,383</td>
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<td>Unique peptides</td>
<td>3,331</td>
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<td>Unique peptides (%)</td>
<td>72.6</td>
<td>73.3</td>
<td>75.8</td>
<td>76.8</td>
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<td>Total proteins</td>
<td>1,076</td>
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<td>836</td>
<td>724</td>
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<tr>
<td>Single hits</td>
<td>518</td>
<td>442</td>
<td>362</td>
<td>312</td>
</tr>
<tr>
<td>Single hits (%)</td>
<td>48.1</td>
<td>46.5</td>
<td>43.3</td>
<td>43.0</td>
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</table>

We next attempted to validate the number of identifications because a large number of the identified proteins were derived from single-hit peptides (43%). Toward this goal, we have purified mitochondria from Jurkat T cells for two independent times and utilized 60 μg each for two separate GeLC-MS/MS analyses (Fig. 2B). We utilized the faster scanning ion trap mass spectrometer, the Finnigan LTQ, with the hope that the ability to validate presumed low-abundant proteins that give rise to single hits will not be limited by the duty cycle of the mass spectrometer. We have generated ∼193,000 and 183,000 MS/MS spectra from the two replicate experiments and analyzed the datasets with the same criteria as the first experiment. Using these results, we were able to validate a total of 599 proteins from the original list of 774. Although we were not able to validate 81 proteins from the first list, due to the high SEQUEST scores and quality of the MS/MS spectra with good consecutive b and y ion series, we are reporting these single hits in the list of proteins potentially present in the mitochondria. All of the proteins that we are reporting corresponded to the correct molecular mass region of the gel except in few cases where the pl and possibly posttranslational modifications of these proteins alter the gel electrophoresis mobility (Fig. 2A). When we examined whether multiple peptides from different gel slices resulted in the single protein identification, we found that except for the 82 proteins that were present in two adjacent gel slices, all of the remaining protein identifications were from a single gel slice (Fig. 2A and Supplemental Table IV).

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three (0.5%) small-molecular-mass proteins (<10 kDa). In this case, the use of longer gradient gel and increasing the sample amount will likely increase the chance of small-molecular-mass protein identification.

The 680 proteins distribute across a wide pI range (4.34–12.3) with a roughly trimodal distribution, typical characteristic of pI histograms for eukaryotic proteins (28). A total of 653 proteins (96%) distribute among pI 4–10 intervals but 27 (4%) proteins have pI greater than 10 and may be not resolvable by the 2D-PAGE separation (Fig. 4B). Interestingly, more than half of the identified proteins in the mitochondrial fraction distribute among pI 8–11 intervals (51.2%), suggesting that subcellular fractionation enriched alkaline proteins (pI 8–12).

About 50% of annotated mitochondrial proteins are theoretically in pI 8–10, consistent with the pI distribution pattern of the currently identified proteins in the mitochondrial fraction. The values of molecular mass and pI reported here do not reflect the mature forms of proteins where a significant number of these are known to be posttranslationally processed. For example, a number of mitochondrial proteins are proteolytically processed during translocation into the mitochondria, and therefore the mature forms differ significantly in their respective molecular mass and pI from gene sequence predictions.

The proteins detected in 2D-PAGE gels are generally hydrophilic with the negative GRAVY values (28–30). For the 680 proteins we identified, their hydrophobicity values vary in the range of –1.6–0.74 (data not shown), indicating that recovery of membrane-bound proteins was not biased as in the case of 2D-PAGE. Additionally, theoretical TMD predicted by SOSUI shows that 120 (17.6%) proteins of the total 680 proteins have one or more predicted TMDs (data not shown), of which 30 proteins have three or more TMDs (Table II). In particular, 14 of the 30 proteins with three or more TMDs are all annotated as integral membrane proteins.

**Functional Classification of Identified Proteins**—Next, we assigned the function of 680 identified proteins based on information available in published literature as well as from the two most comprehensive mitochondrial protein resources: the MitoProteome database (www.mitoproteome.org) and the
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**TABLE II**

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<tr>
<th>Accession ID</th>
<th>Proteins name</th>
<th>Mr</th>
<th>pI</th>
<th>SOSU</th>
<th>Hydrophobicity</th>
<th>Annotated integral</th>
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<td>Cytochrome oxidase c assembly factor (COX15)</td>
<td>46002</td>
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<td>GP:AF037338.1</td>
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<td>76097</td>
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<td>GP:AF085361.1</td>
<td>Mitochondrial carrier homolog 2</td>
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<td>Sodium/potassium-transporting atpase 1 chain precursor</td>
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a Accession numbers in NCBI, SWISS-Prot, or PIR databases.
b TMD and average hydrophobicity prediction by SOSUI.
c Annotated integral membrane proteins (IMP) in SWISS-Prot database.

NCBI’s locus link database (www.ncbi.nlm.nih.gov/LocusLink/). The identified proteins were functionally subdivided into two classes: 466 proteins that can be assigned to known functional classes (69%) and 214 proteins for which no functional information is available (31%). Similar to our findings, a recent study by Taylor et al. revealed 498 functionally known proteins from human heart mitochondria (4). The distribution of the 466 functionally classified proteins is shown in Fig. 5 and is listed in supplemental Table S1. To our knowledge, this additional information of mitochondrial proteins from Jurkat T leukemia cells represent the most comprehensive proteomic data deposited for this cell line.

The Oxidative Phosphorylation Machinery and Metabolism—The oxidative phosphorylation machinery is composed of five electron transport chain complexes, representing a major group of functionally classified mitochondrial proteins. Among the 466 functionally classified proteins, 40 (9%) are components of the five electron transport chain protein complexes from Jurkat T leukemia cells. We also identified the human homolog of one of the three newly discovered complex I subunits in human heart mitochondria: NADH-ubiquinone oxidoreductase ESSS subunit (neuronal protein p17.3).

The bulk of ATP used by many cells to maintain homeostasis is produced by the oxidation of pyruvate in the tricarboxylic acid (TCA) cycle. We identified 25 proteins involved in the TCA cycle (5%). At least one component of each of the seven TCA cycle enzymes were identified along with components of the associated pyruvate dehydrogenase complex and NAD-malic enzymes (Tables S1 and S3). We also identified one enzyme involved in carbohydrate metabolism, methylmalonyl-CoA epimerase, and 10 enzymes of glycolytic pathways. Furthermore, 28 proteins involved in lipid metabolism (6%) were identified, and these include α and β subunits of mitochondrial trifunctional enzymes. We also identified multiple enzymes involved in amino acid and nucleic acid metabolisms: serine hydroxymethyltransferase (both cytosolic and mitochondrial isofrom), glutaminase (kidney isofrom), ornithine aminotransferase, δ-1-pyrrrole-5-carboxylate synthetase, δ-1-pyrrrole-5-carboxylate dehydrogenase, pyrrole-5-carboxylate, and three adenylate kinases.
Cell Rescue, Death, and Defense—Similar to the mitoproteome database, we collectively categorized apoptotic, detoxifying, immune, tumor-related, and DNA repair proteins under the “cell rescue, death, and defense” category. We found a number of crucial proteins that control the apoptotic cell death pathway. For example, programmed cell death-8 (PCD-8), also known as apoptosis-inducing factor (AIF), was the dominant apoptotic protein in this category. In addition, we identified three peptides from Bax protein, which has been reported to be absent or expressed at very low levels in Jurkat T leukemia cells (18, 31). The constitutive expression of Bax protein in the Jurkat T leukemia cell mitochondrial fraction likely provides an explanation for the rapid apoptotic response seen in these cells by the Fas pathway activation (Fig. 7). The expression of Bax in the Jurkat mitochondrial fraction was further confirmed by Western blotting (Fig. 7, A and B, data not shown). In addition, we identified other known regulators of apoptosis, such as BH3 interacting domain death agonist (BID), SMAC protein, bcl-2-like protein 13 (also known as Bcl-Rambo), and B cell receptor-associated protein 31 (BAP31). Interestingly, comparing the cell death-signaling proteins from mitochondria of heart, liver, brain, kidney, and Jurkat leukemia cells, a number of differences in protein distribution were observed (4, 32). For example, Bax protein was detected only in the mitochondrial fraction from Jurkat T leukemia cells. In contrast, endonuclease G, which was present in the mitochondria from liver and heart, was not detected in the Jurkat cells, brain, and kidney. These essential differences may reflect the tissue-specific susceptibility to different apoptotic stimuli.

Protein and Metabolite Transport—The proteins identified include channel proteins of the voltage-dependent anion channels, protein transporters, metabolite transporters, and ion channels (Table S1). Specific proteins listed in Table S1 include proteins that control the exchange of inorganic phosphate, ATP/ADP, and di/tricarboxylates in the mitochondria. Additional proteins from this class include phosphate carrier protein, 2-oxoglutarate/malate carrier protein, Ca2+ -stimulated aspartate/glutamate carrier protein (aralar 1), metaxin-1 and -2, GRPE protein homolog 1, and Sco-1 homolog. GRPE protein homolog 1 cooperates with mitochondrial hsp70 in the import of proteins from the cytoplasm. Similarly, metaxin-1 and -2 can interact with each other and participate in protein transport into the mitochondrion (33). Sco-1 homolog protein
is thought to play a role in either mitochondrial copper transport or insertion of copper into the active site of COX (34). We identified sarcoplasmic reticulum-associated calcium ATPase 1, 2, and 3, which are known to be localized in the area of contact between the sarcoplasmic reticulum and the outer mitochondrial membrane (35). We also found sodium/potassium-transporting ATPase \( H^{+}/K^{+} \)-1 chain precursor, which was previously detected in human heart mitochondria (4).

**Redox Proteins**—In this category, we identified 14 redox-related proteins, which include NAD(P) transhydrogenase, peroxiredoxin (three isoforms), quinone oxidoreductase, cytochrome b5 (two isoforms), NADH-cytochrome b5 reductase, protein disulfide isomerase precursor (three isoforms), quinone oxidoreductase, and manganes superoxide dismutase (MnSOD) (Table S1). We also found kidney dicarbonyl reductase (highly expressed in kidney), which is similar to the mammalian diacetyl reductase and L-xylulose reductase.

**Signaling Proteins**—By MS analysis of the Jurkat T cell mitochondria, we found a striking diversity of signaling proteins that include small GTPases, heterotrimeric G-proteins, kinases/phosphatases, and other proteins that have a role in intra- and intercellular communication (Table S1). Most of the 10 identified small GTPase members belong to the Ras family (Rab). Six of them were previously reported in the human heart mitochondrial proteome analysis (4). Ran-binding protein 2 and Rho GDP-dissociation inhibitor 2 were also detected in this analysis. In addition to the small GTPase members, four heterotrimeric G-protein \( \alpha \)-subunits were found in the mitochondria, with the G12 \( \alpha \)-subunit being particularly abundant based on the number of peptides identified. Moreover, we identified several members of serine/threonine kinases/phosphatases including the serine/threonine protein phosphatase 1, serine/threonine protein phosphatase 2a, \( \alpha \) isoform, serine/threonine-protein kinase plk, rho-interacting serine/threonine kinase 21, cyclin-dependent kinase 1, and calcineurin subunit A. Some of these proteins have been shown to be involved in mitochondrial apoptotic pathways. Interaction of serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) with certain regulators of the Bcl-2 family is critically involved in the control of apoptosis (36). For example, it has been shown that C2-ceramide mediates apoptosis by the specific activation of a mitochondrial
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PP2A, which dephosphorylates Bcl2 and inhibits its anti-apoptotic function (37). In addition, cyclin-dependent kinase 1 (Cdc2) has been shown to phosphorylate BAD and cause apoptosis (38). Thus, it is likely that the presence of these kinases and phosphatases in the mitochondria is biologically relevant.

Protein Destination and Proteases—Proteins identified in the “protein destination” category are involved in protein complex assembly, protein modification, protein targeting, and protein stabilization. We found cytochrome oxidase assembly factor (COX15), which might be involved in electron transport chain assembly in mitochondria (18). We identified six subunits of the inner and outer mitochondrial membrane translocases, TIM and TOM, which function in the protein import into the mitochondria. The proteins involved in protein stabilization, such as the heat-shock proteins and three cytoplphilins A, B, and F, were found. We also identified protein-modifying enzymes, ubiquitin-like protein SUMO-1-conjugating enzyme (Ubc9), and ubiquitin-protein ligase e3a. Ubiquitin-like protein SUMO-1-conjugating enzyme catalyzes the covalent attachment of ubiquitin-like protein SUMO-1 to other proteins (39). Consistent with our findings, it has been recently shown that in addition to the nucleus, Ubc9 and SUMO-1 are localized in mitochondria in COS7 cells. SUMO-1 has been shown to conjugate to mitochondrial substrate(s) and participate in mitochondrial fission (39). In addition, we also found a number of proteases such as the endopeptidase La homolog precursor, the lon protease homolog, the caax prenyl protease 1 homolog, and sentrin-specific protease 5. Caax prenyl protease 1 homolog proteolytically removes the C-terminal three residues of farnesylated and geranylated proteins (40). On the other hand, sentrin-specific protease 5 releases SUMO-1 from its precursor sequence (41).

Structural Proteins—Mitochondrial fraction was found to contain a large number of cytoskeleton proteins, particularly those of actin family members. In addition to α and β actin, we also found some actin binding or regulatory proteins, including α adducin, emerin, ezrin (p81), and coflin. We found 26 and 53 peptides for the nonerythrocytes α and β spectrins, respectively, in addition to six peptides for erythrocyte spectrin (Table S1). Numerous proteins of microtubule cytoskeleton and intermediate filaments were identified by MS analysis of mitochondria (Table S1). In our study, we found tubulin β-1 chain, tubulin α-2 chain, tubulin α-6 chain, and vimentin. We also identified three septin family proteins in purified mitochondria, including septin 1, septin 2, and septin 7. It is likely that these proteins tether mitochondria to cell cytoskeleton as described previously (32). This is also supported by the fact that tubulin is an inherent component of mitochondrial membranes, and it could play a role in apoptosis via interaction with the permeability transition pore (42). However, we cannot conclusively rule out the possibility that there is a trace amount of contaminant cytoskeletal proteins in our preparation even though our preparation is devoid of the abundant cytosolic protein LDH.

RNA, DNA, and Protein Synthesis—In this major category, we found 31 proteins from the mitochondrial ribosomal proteins of the 39S and 29S subunits. Furthermore, we identified DNA-directed RNA polymerase, mitochondrial elongation factor G1, tryptophanyl-tRNA synthetase, elongation factor ts, and elongation factor tu (Table S1). In addition to mitochondrial protein synthesis machinery, LC-MS/MS analysis identified multiple cytoplasmic ribosomal proteins (19 proteins) and factors that regulate translation (Table S1). A number of RNA binding proteins and heterogenous nuclear ribonucleoproteins (hnRNPs) were also detected. It is widely known that most mitochondrial protein are encoded in the nucleus, synthesized by free cytosolic ribosomes, and translocated into mitochondria posttranslationally. However, evidence suggests that some proteins may be imported cotranslationally (43). Because outer mitochondrial membranes contain receptors specific for ribosomes, direct interaction between mitochondria and cytosolic ribosomes support cotranlational import (43). In support of the association of hnRNP proteins with mitochondria, it has been shown that hnRNP K protein translocates into the mitochondria and interacts with multiple mitochondrial transcripts within this organelle (44). Proteome analysis of Jurkat T leukemia cells identified the hnRNP K protein during apoptosis (45). Involvement of several other hnRNP family proteins in apoptosis has been reported (45).

Nonmitochondrial Proteins—A large number of proteins detected in our mitochondrial preparation have been described so far for other subcellular compartments. A majority of the nonmitochondrial proteins identified here in this study are cytoplasmic proteins according to database information and prediction by bioinformatics tools. A similar result was also obtained from MS analysis of human heart and mouse liver mitochondria (4, 46). We propose that the association of cytoskeleton proteins, endoplasmic reticulum and its associated proteins, cytoplasmic signaling proteins, and ribosomal proteins with mitochondria is likely to be physiologically relevant. Consistent with this notion, in our mitochondrial preparations, we identified several glycolytic enzymes including the hexokinase I. This enzyme has been previously shown to be localized in outer mitochondrial membrane and possibly to modulate glycolysis (47). Among the other nonmitochondrial proteins, we also found four lysosomal proteins, two peroxysomal proteins, some Golgi proteins, and some vesicle-associated membrane proteins, a large majority of which have been reported in other mitochondrial preparations (32, 46). This may indicate functional association of mitochondria with other cellular compartments rather than contaminations. The intimate association between mitochondria and the nucleus, coupled with electrostatic effects, may explain the presence of nuclear proteins in our preparation and also shown in other preparations. In addition to histones, we also found a number of nuclear pore complex proteins and helicase enzymes. A
recent proteomic survey of highly purified yeast mitochondria by Sickmann et al. (5) identified 750 proteins of which 436 had been previously shown to be mitochondrial proteins. Similar to what is reported here, 314 proteins identified in the yeast mitochondrial study are proteins from multiple subcellular compartments.

**Prediction of Subcellular Localization**—We classified all of the 680 identified proteins in our mitochondrial preparation into two categories: “known functional group” and “unknown functional group” (Tables S1 and S2). About 31% of the 680 proteins identified here have not been assigned to a functional group. For these proteins with no data available, we utilized two bioinformatic tools, PSORT II and TargetP, to predict their subculture localization (23, 24). We found that the newer version of PSORT II can predict proteins into 17 different subcellular locations. The TargetP predictor has a more limited prediction scope than PSORT II and therefore we are showing the data from the PSORT II prediction only. To estimate the confidence of these predictions, we first processed sequence of known mitochondrial proteins. We found the highest agreement with 62.0% for PSORT II (146 of 240 proteins). As shown in Fig. 6, PSORT II predicted a putative mitochondrial localization for the additional 25% of 214 proteins. Thus, 58 additional proteins were predicted, and this allows the total number of mitochondrial signal-containing proteins to be 285. Furthermore, 77 proteins were predicted for cytoplasmic localization (37%), 41 proteins were predicted for nuclear localization (19%), 26 proteins for endoplasmic reticulum (12%), and 15 proteins for other cellular compartments (7%). While several bioinformatics tools are currently available for detecting mitochondrial targeting sequence, such predictions still suffer from poor sensitivity and specificity. Clearly, additional studies are needed to fully validate the prediction of subcellular localization of all these unknown proteins.

**CONCLUSION**

In this study, with the combination of differential centrifugation, sucrose density gradient, GeLC/MS/MS, and multiple validation experiments, we identified 599 mitochondria and mitochondria-associated proteins. In addition, we are including the list of 81 proteins identified from the first experiment in the list due to their quality of the spectra, fitting the gel band molecular mass constraint, as well as the SEQUEST scores. Similar to the recent replicate analysis of human heart mitochondria, we found that each of the replicate samples identified 135 common proteins with a number of unique proteins identified from each experiment (48). We believe that this incomplete coverage in replicate analyses is due in part to the overall complexity of proteins and wide dynamic range of the mitochondrial proteome. Thus, it is conceivable that even the faster scanning mass spectrometers cannot completely identify all the available peptides from the replicate samples. We have included the list of 81 proteins identified only with a single peptide hit in our final list as this information may be crucial for additional investigation and further validation experiments.

This approach clearly utilizes significantly less starting material and thus reduced the protein separation and MS time when compared with a number of other proteomic approaches (9–13). Using this simple strategy, we demonstrate that efficient identification mitochondrial proteins with a wide range of biochemical characteristics can be achieved. However, the very-low-abundant proteins in mitochondria may escape identification by current strategy. Assuming that current detection limit of LCQ-DECA is at 10 fmol, \(~6 \times 10^9\) molecules must be present in the samples for protein identification. Thus, from our starting number of \(~0.75 \times 10^7\) cells, which provided 40 \(\mu\)g of mitochondrial proteins, we estimate that we could detect proteins that are expressed at 800 copies per cell. To identify a greater complement of the mitochondrial proteins including the proteins that are expressed at a much lower than 800 copies per cell, sample scale-up as well as an additional increase in sensitivity of the mass spectrometer are required.

Moreover, to comprehensively identify proteins that are expressed in cells, many additional factors influence the success of the study; proteins solubility, type of chromatographic column for peptide separation, and sensitivity and duty cycle of mass spectrometers. In terms of specific proteins with different solubility, optimization of detergent and buffer conditions as well as the differential extraction of proteins with at least two or more detergents may be required. Furthermore, for the identification of very hydrophobic proteins, both C-18 and C-8 reverse-phase chromatographic columns could be used for the same sample. Finally, with the help of highly sensitive and faster scanning linear ion trap mass spectrometers such as the LTQ (Thermo Finnigan), approximately two times more proteins can be identified (unpublished results). Future studies and optimization steps are required to identify mitochondrial proteins comprehensively from different tissues and cells.

Similar to the genomics community, we anticipate that large-scale proteomic datasets in a standardized format will be available to investigators in the future. We envision that the availability of subcellular proteomes will allow numerous investigators to perform comparative studies and further understand tissue-specific differences exhibited in human. There are many tissue-specific differences in response to hormonal and external stimuli exhibited in vivo and some of these are very well characterized (49, 50). For example, it is known that apoptotic-inducing anti-Fas IgM triggers apoptosis in the liver but not in the heart in mouse, although both tissues express Fas receptors (50). Why these differences are exhibited in the liver and heart is not well understood. We anticipate that comprehensive characterization of proteomes from different cell and tissue types will allow us to better understand how tissue-specific differences are exhibited. Thus, future pro-
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teomic studies are expected to help understand essential differences that are present in physiological and pathological conditions.

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