Mortalin: A Protein Associated With Progression of Parkinson Disease?

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Abstract

Parkinson disease (PD) is a progressive neurodegenerative disorder that is considered to affect the brainstem at its early stages and other brain regions, including the limbic system and isocortex, in advanced stages. It has been suggested that PD progression is characterized pathologically by the spreading of Lewy body deposition. To identify novel proteins involved in PD progression, we prepared subcellular fractions from the frontal cortex of pathologically verified PD patients at different stages of disease and Lewy body deposition and from age-matched controls. Protein expression profiles were compared using a robust quantitative proteomic technique called isobaric tagging for relative and absolute quantification in conjunction with mass spectrometry. Approximately 200 proteins were found to display significant differences in their relative abundance between PD patients at various stages and controls. Gene ontology analysis indicated that these altered proteins belonged to many categories (e.g., mitochondrial function and neurotransmission) that were likely critically involved in the pathogenesis of PD. Of those, mortalin, a mitochondrial protein, was decreased in the advanced PD cases and was further validated to be decreased using independent techniques. These results suggest a role for mortalin in PD progression.

Key Words: Frontal cortex, Isobaric tagging for relative and absolute quantification, Lewy body, Mortalin, Parkinson disease progression

INTRODUCTION

Parkinson disease (PD) is a relentlessly progressive neurodegenerative disorder that afflicts more than 1 million people in North America alone (1, 2). Clinically, PD patients present with bradykinesia, resting tremor, muscular rigidity, and gait abnormalities, as well as nonmotor symptoms, including anosmia, constipation, depression, autonomic failure, and cognitive dysfunction (1, 2). Pathologically, PD is characterized by a profound and relatively selective loss of neurons in the brainstem, including dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc), and the presence of cytoplasmic inclusions called Lewy bodies (LBs) in surviving neurons (1). More recently, Braak et al (3) have suggested that as the disease advances, it is invariably accompanied by spreading of LBs from the brainstem to limbic system and, eventually, to the isocortex.

The staging system of Braak et al for PD is based on α-synuclein immunohistochemical analysis of aggregated α-synuclein, a major component of LBs. To date, the physiological roles of α-synuclein have not been fully characterized. Furthermore, the mechanism by which α-synuclein and other neuronal components such as ubiquitin, phosphorylated neurofilaments, and heat shock proteins gradually transform into insoluble LBs remains to be elucidated (1, 4). Additionally, although mitochondrial and proteasomal dysfunction and oxidative stress are likely involved in PD pathogenesis, it remains to be defined as to how aggregated α-synuclein induces neuronal death in PD (1, 2, 4).

In this study, we used a robust shotgun proteomic approach in conjunction with an isotope labeling technique, isobaric tagging for relative and absolute quantification (iTRAQ) (5, 6), to compare protein profiles quantitatively in the frontal cortex of PD patients at different stages of disease progression and age-matched controls. This investigation identified many novel proteins with quantitative expression differences in the samples from different PD stages. Of these proteins, mortalin (also known as stress-70 protein, mitochondrial precursor, or GRP75) was also found to be decreased in late stages of PD cases by Western blot and 2-dimensional (2D) gel electrophoresis, thereby validating the proteomics results.

MATERIALS AND METHODS

Characterization of Human Brain Tissue

Samples of the frontal cortex (middle frontal gyrus) from each case were collected at autopsy at Emory University School of Medicine, the University of Michigan, and the University of Washington School of Medicine. Four groups of samples (20 cases) were analyzed: age-matched controls (n = 5), PD with LBs located in the brainstem only
(PD-brainstem; n = 5), PD with LBs observed in the brainstem and limbic system (PD-limbic; n = 5), and PD with LBs in the frontal cortex in addition to the brainstem and limbic system (PD-isocortex; n = 5). Age-matched normal controls were individuals who did not have a diagnosis of neurologic disease, were not taking medications prescribed for neurologic diseases, and whose neuropathologic examination revealed age-related changes only. All patients had been diagnosed with PD (with and without cognitive impairment) according to the National Institute of Neurological Disease criteria, and final diagnoses were established by neuropathologic examination according to established criteria (7, 8). All patients had a PD diagnosis first, indicating that dementia with LB disease cases were excluded from the study. All subjects were age-, sex-, and postmortem interval (<12 hours)–matched.

Biochemical Cellular Fractionation

Frozen samples were rapidly thawed in ice-cold homogenization buffer (0.32 mol/L sucrose, 20 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.5, 1 mmol/L phenylmethylsulfonyl fluoride, phosphatase inhibitors [0.2 mmol/L Na3VO4 and 1 mmol/L NaF], and protease inhibitor cocktail from Sigma, St Louis, MO) and then disrupted with a glass-Teflon homogenizer (Wheaton, Millville, NJ) by 10 gentle up-and-down strokes. The protein concentrations of the homogenates were determined using a Pierce bicinchoninic acid assay protein assay kit (Rockford, IL). Equal amounts of proteins from 5 individual samples in each group were pooled. Subcellular fractionation was then performed as previously described (9, 10) with minor modifications. Briefly, the pooled homogenate was first centrifuged at 100 x g for 30 seconds to remove large cell debris. The supernatant was collected and centrifuged for 10 minutes at 800 x g to separate the crude nuclear pellet and the cytosolic supernatant. The cytosolic supernatant was further centrifuged at 10,000 x g for 15 minutes to obtain the mitochondria-enriched pellet (mitochondrial fraction) and the cytosol-enriched supernatant (cytosolic fraction). The mitochondria-enriched pellet was resuspended in the sample buffer consisting of 6 mol/L urea, 0.05% sodium dodecyl sulfate (SDS), 5 mmol/L EDTA, and 50 mmol/L Tris-HCl (pH 8.5). The crude nuclear pellet from the second centrifugation was resuspended in a nuclear extraction buffer (20 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, pH 7.9, 0.3 mol/L KCl, 1.5 mmol/L MgCl2, 20% glycerol, and 0.1% Triton X-100). After 30 to 40 minutes of gentle shaking, the suspension was centrifuged at 14,000 x g for 10 minutes. The resulting supernatant, representing the nuclei-enriched fraction, was subsequently desalted using a PD-10 column (Amersham/GE, Piscataway, NJ), dried in a centrifugal vacuum concentrator, and redissolved in the sample buffer (6 mol/L urea, 0.05% SDS, 5 mmol/L EDTA, 50 mM Tris-HCl, pH 8.5).

iTRAQ Labeling and Sample Separation With 2D Chromatography

A comparison of the relative abundance of protein levels in each enriched fraction in the 4 groups (i.e. controls vs PD-brainstem, PD-limbic, and PD-isocortex) was achieved using the iTRAQ labeling technique that is routinely used in our lab (11). In this study, for each enriched fraction, 100 µg of proteins from each group was digested with trypsin in parallel and then labeled with iTRAQ reagents (Applied Biosystem, Foster City, CA) according to the manufacturer’s protocol. The labeled digests from the 4 groups were combined together and further separated into 9 fractions using a strong cation exchange PolySulfoethyl A column (Poly LC, Columbia, MD). Strong cation exchange–fractionated peptides from each fraction were then dissolved in 0.5% trifluoroacetic acid and separated using reverse-phase chromatography. Nanocapillary liquid chromatography (LC) was performed using the LC Packings UltiMate with Famos autosampler and Switchos automated switching valve (LC Packings, Sunnyvale, CA). Samples were loaded onto a capillary precolumn cartridge ( Dionex, Sunnyvale, CA). The trap column was washed with mobile phase A containing 2% acetonitrile and 0.1% trifluoroacetic acid in high-performance LC water. The flow rate was set at 0.4 µL/min. The sample was then loaded onto a 15 cm x 100-µm ID Magic C18 3 µm, 100-A0030A packing capillary LC column (Michrom Bio-Resources, Inc., Auburn, CA). The gradient run was from 5% mobile phase B (80% acetonitrile, 0.08% trifluoroacetic acid in high-performance LC water) to 90% mobile phase B for 85 minutes. The eluted gradient was mixed with 7 mg/mL recrystallized α-cyano-4-hydroxycinnamic acid (Sigma) in 60% acetonitrile, 2.6% (5 mg/mL) ammonium citrate with the internal standard (4700 Mass Standard Kit, Applied Biosystems, Foster City, CA) and spotted onto a stainless steel MALDI plate with the Probot (LC Packings). Samples were spotted at 5-second intervals using a 24 x 24-array pattern for a total of 576 spots per plate. The experiments starting from the sample preparation were repeated 3 times. In total, 162 MALDI plates were spotted and analyzed by a 4700 Proteomic System (Applied Biosystems).

Analysis of Enriched Fractions With MALDI TOF/TOF Tandem Mass Spectrometry

Quantitative tandem mass spectrometry (MS/MS) analysis was performed using a 4700 Proteomics Analyzer with TOF/TOF Optics (Applied Biosystems). Mass spectrometry reflector positive ion mode with automated acquisition of 800 to 4,000 m/z range was used with 1,000 shots per spectrum. A maximum of 15 peaks were selected per spot, with a minimum signal-noise ratio of 50 and cluster area of 500. More than 36,000 precursors were selected and submitted for MS/MS, where a positive ion mode with collision induced-dissociation cell on and 1 kV collision energy were used, and 3,000 shots were accumulated per spectrum. A total of 576 MS and more than 1,200 MS/MS spectra were acquired for each spotted plate. The MS/MS spectra were searched against the International Protein Index human protein database (version 3.18) using Mascot algorithm (Matrix Science, Boston, MA) for peptide and protein identification as described (11). Proteins identified by decoy database were eliminated. Protein quantification was achieved by averaging weighted iTRAQ ratios of all corresponding peptides using the GPS Explorer (Applied Biosystems, Foster City, CA).
Western Blotting

Briefly, 10 μg of protein from the enriched fractions was subjected to 8% to 16% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). The membrane was blocked and incubated overnight with a mouse anti-mortalin/GRP75 antibody (Stressgen/Assay Designs, Ann Arbor, MI) at a dilution of 1:2500 or with a β-actin antibody (Abcam, Cambridge, MA) as the loading control. The primary antibody was then detected using a horseradish peroxidase–conjugated secondary antibody at a dilution of 1:20000. Blots were developed using the enhanced chemiluminescence Western blotting analysis system (Amersham/GE). Protein expression differences were quantified using the Quantity One software (Bio-Rad, version 4.63) and normalized by β-actin expression. The experiment was repeated at least 3 times. Changes between groups were analyzed by 1-way analysis of variance and Newman-Keuls test using GraphPad Prism 4.0 (San Diego, CA). p < 0.05 was accepted as significant.

2D Gel Electrophoresis

Cytosol-enriched fractions from human frontal cortex were desalted and diluted in rehydration buffer (Bio-Rad). Briefly, 100 μg of proteins in 200 μL of rehydration buffer was loaded onto an 11-cm, pH 4 to 7 immobilized pH gradient strip (Bio-Rad) by active hydration at 50 V for 12 hours. Isoelectric focusing was conducted in Protean IEF Cell (Bio-Rad) for a total of 50,000 Vh at 20°C. After focusing, immobilized pH gradient strips were sequentially equilibrated in equilibration buffers I (6 mol/L urea, 0.375 mol/L Tris, pH 8.8, 2% SDS, 20% glycerol, 2% dithiothreitol) and II (6 mol/L urea, 0.375 mol/L Tris, pH 8.8, 2% SDS, 20% glycerol, 2.5% iodoacetamide) and then subjected to the second-dimension separation on a Precast 8% to 16% Tris-HCl Criterion SDS-polyacrylamide gel electrophoresis gel (Bio-Rad) at 100 V for 10 minutes and then 160 V for 1 hour. Gel electrophoresis experiments were performed in duplicates; 1 gel was stained with silver, and the other gel was electrotransferred to polyvinylidene fluoride membranes and blotted with an anti-mortalin/GRP75 antibody (1:20000; Stressgen/Assay Designs).

RESULTS

Quantitative Analysis of Protein Profiles in 3 Cellular Fractions

All current MS-based proteomics are biased toward identification of proteins of high abundance (14, 15); thus, it is critical to fractionate tissue homogenates to achieve a deeper analysis of the cellular proteome through enrichment of low-abundance proteins. Moreover, this approach increases the likelihood of identifying the proteins whose total cellular levels remain the same but whose relative levels in specific cellular compartments change during processes. In this study, cytosol-, mitochondria-, and nuclei-enriched fractions were prepared from the pooled frontal cortex homogenate of 5 PD-brainstem patients, 5 PD-limbic patients, 5 PD-isocortex patients, and 5 normal age-matched controls, respectively. For every comparison, each of the 4 groups was labeled with a specific iTRAQ reagent, and the samples were then mixed for relative quantifications. After running the experiment in triplicate, the identification results of the 3 independent runs were combined. A total of 1,864 nonredundant proteins were identified from the combined fractions. Among them, 940 proteins were identified in the cytosol-enriched fraction, 885 proteins were identified in the mitochondria-enriched fraction, and 1,255 proteins in the nuclei-enriched fraction. Proteins identified by a single peptide should be considered provisional according to the guidelines of most proteomics experts (16). A full list of proteins identified in human cortex by more than 2 peptides and their characteristics is reported separately along with proteins identified with another fractionation method (17).

One of the major issues in qualitative and quantitative proteomics is low reproducibility owing to variation in ionization of peptides and sampling of ionized peptides when identification is achieved with MS/MS analysis (14). For these reasons, proteins identified in each experiment need to be combined. Similarly, proteins quantified in each experiment also need to be pooled. Among the total proteins identified, 649 nonredundant proteins displayed significant changes in relative abundance between PD and control groups. One hundred ninety-nine of them were identified by 2 or more peptides. A complete list of these proteins identified by more than 2 peptides and by a single peptide can be found in Supplemental Appendix 1A and 1B, respectively.

All proteins identified and quantified need to be validated before pursuing their biologic functions. Currently, this cannot be done in high-throughput fashion. One way of approaching this obstacle is to repeat protein-profiling experiments and focus on the proteins that can be consistently quantified and identified. To this end, among all the proteins identified with quantitative changes, 256 (33.4%) were identified in at least 2 independent experiments. These proteins are reported in Supplemental Appendix 2. In the cytosol-enriched fraction, 63 proteins were found to decrease in the PD-brainstem group as compared with the control group (23 were identified in at least 2 experiments). Of the 63 proteins, 30 remained decreased in the PD-limbic group, whereas the expression of most of the other 33 proteins...
returned to control levels. We identified 14 proteins that were decreased only in the PD-limbic group and 24 proteins that were decreased only in the PD-isocortex group. More interestingly, 15 proteins consistently, and some of them progressively, decreased in the PD groups, including mortalin, glutathione S-transferase Mu 3 (GSTM3), and excitatory amino acid transporter 2. In contrast, the levels of 69 proteins were increased in the PD-brainstem group, 74 proteins in the PD-limbic group, and 73 proteins in the PD-isocortex group. Among them, only 33 proteins showed consistent increases in all 3 PD groups (Supplemental Appendix 1, 2; Table). Similar changes in protein expression profiles were also observed in the mitochondria- and nucleic-enriched fractions (Supplemental Appendix 1, 2).

In many cases, the list of differentially expressed proteins/genes is not sufficient for accurate inference of the underlying biology (13). To identify relevant biologic processes or functions from the protein expression data, we used biologic category analysis via the cumulative hyper-geometric distribution method of determining enhanced GO categories (13). This approach uses protein lists and identifies GO categories by evidence of overrepresentation of significant proteins. The 199 significantly changed proteins identified by 2 or more peptides (i.e. the proteins were identified with high confidence) were classified in accordance with their distribution into cellular components and biologic processes based on GO analysis and annotations (Fig. 1). The top-ranked GO biologic process categories or most statistically overrepresented biologic functions of the proteins identified include (central) nervous system development, regulation of neurotransmitter levels/regulation of axon extension/synaptic transmission, synaptic vesicle exocytosis/glutamate transport/endocytosis, electron transport/proton transport, small guanosine triphosphatase-mediated signal transduction, chromatin silencing/histone deacetylation, and the establishment of protein localization/protein export from nucleus and endoplasmic reticulum to Golgi vesicle-mediated transport.

The Table shows selected proteins from the top-ranked GO categories. They were also identified in at least 2 independent experiments and can be divided into 3 types: 1) those known to have potential roles in PD or other neurodegenerative disease pathogenesis, for example, mortalin (10), apolipoprotein E (18), huntingtin interacting protein 2 (19, 20), and GSTM3 (21); 2) those known to be important in central nervous system (CNS) function, but previously not

### TABLE. Top-Ranked Proteins (GO analysis) With Changes Between PD Groups and Controls

<table>
<thead>
<tr>
<th>IPI No.</th>
<th>Protein Name/Function</th>
<th>Peptides Found*</th>
<th>PD-B/Ctrl†</th>
<th>PD-L/Ctrl†</th>
<th>PD-I/Ctrl†</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPI00300020</td>
<td>Excitatory amino acid transporter 2/essential for terminating the postsynaptic action of glutamate by rapidly removing released glutamate from the synaptic cleft</td>
<td>4</td>
<td>0.62 ± 0.04</td>
<td>0.47 ± 0.20</td>
<td>0.76 ± 0.22</td>
</tr>
<tr>
<td>IPI00246975</td>
<td>GSTM3/may govern uptake and detoxification of both endogenous compounds and xenobiotics at the testis and blood-brain barriers</td>
<td>4</td>
<td>0.64 ± 0.35</td>
<td>0.58 ± 0.09</td>
<td>0.49 ± 0.36</td>
</tr>
<tr>
<td>IPI00021370</td>
<td>Huntingtin interacting protein 2/ubiquitin-conjugating enzyme E, 2–25 kd/mediates the selective degradation of short-lived and abnormal enzymes such as huntingtin and p53 by ubiquitination</td>
<td>2</td>
<td>2.59 ± 0.55</td>
<td>2.70 ± 0.88</td>
<td>3.21 ± 0.00</td>
</tr>
<tr>
<td>IPI00007765</td>
<td>Stress-70 protein, mitochondrial precursor/mortalin/implicated in the control of cell proliferation and cellular aging. May also act as a chaperone</td>
<td>5</td>
<td>0.66 ± 0.01</td>
<td>0.74 ± 0.17</td>
<td>0.49 ± 0.15</td>
</tr>
<tr>
<td>IPI00554760</td>
<td>Tenascin-R precursor, isoform 2/neural extracellular matrix protein involved in interactions with different cells and matrix components</td>
<td>3</td>
<td>1.39 ± 0.29</td>
<td>1.56 ± 0.11</td>
<td>1.81 ± 0.25</td>
</tr>
<tr>
<td>IPI00218848</td>
<td>ATP synthase e chain, mitochondrial/one of the chains of the nonenzymatic component (CF0) subunit of the mitochondrial ATPase complex</td>
<td>2</td>
<td>0.66 ± 0.60</td>
<td>0.58 ± 0.17</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>IPI00216477</td>
<td>Myelin basic protein, isoform 5/myelin membrane formation and stabilization</td>
<td>9</td>
<td>2.25 ± 0.09</td>
<td>2.07 ± 0.22</td>
<td>2.04 ± 0.12</td>
</tr>
<tr>
<td>IPI00382551</td>
<td>NAD-dependent deacetylase sirtuin 2, isoform 2/involved in the control of mitotic exit in the cell cycle, probably via its role in the regulation of cytoskeleton</td>
<td>2</td>
<td>2.38 ± 0.80</td>
<td>1.88 ± 0.81</td>
<td>1.86 ± 0.62</td>
</tr>
<tr>
<td>IPI00031169</td>
<td>Ras-related protein Rab-2A/required for protein transport from the ER to the Golgi complex</td>
<td>3</td>
<td>0.82 ± 0.31</td>
<td>0.61 ± 0.13</td>
<td>0.64 ± 0.18</td>
</tr>
</tbody>
</table>

**Protein names are from the IPI human protein database (version 3.18). Protein functions are from the UniProtKB/Swiss-Prot database.**

* Peptides found denote the sum of all different peptides found and relatively quantified from the proteins shown in 3 replicate experiments.

† The ratio shown is the mean of at least 2 independent experiments ± SE.

ATP, adenosine triphosphate; Ctrl, Control; ER, endoplasmic reticulum; GO, gene ontology; IPI, International Protein Index; NAD, nicotinamide adenine dinucleotide; NDRG2, N-Myc downstream-regulated gene 2; PD, Parkinson disease; PD-B, PD-brainstem; PD-L, PD-limbic; PD-I, PD-isocortex.
associated with PD, for example, excitatory amino acid transporter 2 (22), nicotinamide adenine dinucleotide–dependent deacetylase sirtuin 2 (23, 24), and N-Myc downstream-regulated gene 2 (25); 3) those known to be important in various cellular processes but not known to be related to the CNS, for example, adenosine triphosphate synthase e chain and calnexin.

**Validation of Mortalin by Western Blot and 2D Electrophoresis**

Because proteins can be identified incorrectly due to incomplete human protein databases, candidate proteins identified by a high-throughput proteomic analysis need to be validated before their biologic roles are investigated extensively. In our first step toward verifying the proteins most likely to have biologic importance in PD, we chose mortalin, the relative levels in cytosol-enriched fractions of which were significantly decreased in all 3 PD groups (Table). In addition, our previous study identified mortalin as a protein that is substantially reduced in the mitochondrial fraction isolated from the SNpc of PD patients compared with age-matched controls (10). Moreover, manipulations of its level in cultured DAergic neurons resulted in significant changes in sensitivity to PD phenotypes via pathways involving mitochondrial and proteasomal function as well as oxidative stress. Other studies have also indicated a critical role for mortalin in stress response, cell proliferation, and survival (26). As shown in Figure 2A, the expression of mortalin was detected in all 3 cellular fractions by Western blotting analysis and seemed to be decreased in the cytosol-enriched fractions of samples from PD groups, consistent with our proteomic analysis results (Fig. 2B).

Because mortalin assumes different isoforms (26), it is possible that these isoforms may be regulated differently. To determine whether 1 or more specific mortalin isoforms decreases as the disease progresses in PD, we further analyzed protein expression levels of mortalin isoforms in the cytosol-enriched fraction using 2D gels. Four mortalin isoforms were observed in controls, but it seemed that only 1 isofrom could be detected in PD groups, and its level decreased as compared with controls (Fig. 3). The protein level of this isofrom decreased dramatically in the PD-limbic group, whereas in the same group, the total protein level detected by the regular Western blotting did not decrease as much (Fig. 2), indicating that the relative amounts of different isoforms might be regulated differentially. Nonetheless, taken in concert, the 2D results largely corroborated the results obtained by iTRAQ and regular Western blotting analyses, and also indicated that all isoforms were affected as the disease progressed.

We had previously demonstrated that mortalin was associated with both soluble α-synuclein and the multifunctional protein DJ-1 (27), although it did not colocalize to LBs in the remaining neurons in the SNpc (10). To explore the relationship between mortalin and cortical LBs, we also performed immunohistochemical and confocal analyses on paraffin-embedded human frontal cortex samples from a different set of PD subjects. The results seemed to suggest that mortalin was present in neurons of the human frontal cortex but not in LBs that were positive for α-synuclein immunostaining (data not shown). More definitive studies are needed, however, to confirm this observation.

**DISCUSSION**

Here, we report a list of candidate proteins that might be important in PD progression associated with the spreading of LBs from the brainstem to the limbic system and, eventually, the isocortex. Altogether, approximately 200 proteins that we identified with high confidence displayed significant differences in their relative abundance between PD patients at various stages of disease progression and controls. The change in expression of 1 of these proteins, mortalin, was further validated using Western blotting and 2D gel electrophoresis.

It is important to stress that quantitative changes of protein profiles were detected initially with pooled samples,
and results from each experiment were combined. This is in contrast to most conventional biologic experiments in which individual variability is emphasized. Our method of data analysis was used because of limitations of current MS technology, that is, variation in ionization of peptides and sampling of ionized peptides that result in variability in a list of protein identified when an identical sample is run multiple times (14, 28). Isotope labeling largely circumvents the first limitation, but overlap of the proteins identified (and quantified) is between 25% and 60% depending on the extent of peptide separation and characteristics of the mass spectrometer used (10, 11). Therefore, when a protein is seen in 1 experiment but not the other, it is not possible to know whether the protein is not present or simply not detected. Thus, during the last few years, we have adopted the following strategy for this type of proteomics discovery (11). First, extensive analysis is performed with well-characterized samples that are labeled with isotopes. With this approach, we can be certain that when a protein is differentially expressed, it has to be regulated at least in a subset of patients. After candidate proteins are identified, 1 or more candidate proteins are validated with alternative means with individual samples to ascertain individual variability of the protein(s) of interest.

![Figure 2](http://jnen.oxfordjournals.org/)

**FIGURE 2.** Expression analysis of mortalin by Western blotting. (A) Western blotting analysis of mortalin abundance in the cytosol (cyt)-, mitochondria (mt)-, and nuclei (nu)-enriched fractions. β-actin was used as a loading control. This is representative of at least 3 independent experiments. (B) Quantification of the band signals indicated a significant decrease in mortalin levels in PD groups in the cytosolic fraction, corroborating the results obtained by iTRAQ analysis. Open bars = ratios from the iTRAQ analysis. The primary full-length blots can also be found in Supplemental Appendix 3. *p < 0.05 compared with controls. CTRL, control; PD, Parkinson disease; PD-B, PD-brainstem; PD-I, PD-isocortex; PD-L, PD-limbic.

![Figure 3](http://jnen.oxfordjournals.org/)

**FIGURE 3.** Two-dimensional electrophoresis analysis of mortalin isoform levels. Cytosol-enriched fractions from different groups were analyzed in 2D gels and visualized by immunostaining using an antibody against mortalin or β-actin as a control. Representative gels for detailed immunoblots of mortalin are shown. Four mortalin isoforms were observed in controls (A), but it seemed that only 1 isoform could be detected in the Parkinson disease (PD)-brainstem (B), PD-limbic (C), and PD-isocortex (D) groups, and its level decreased as compared with controls, largely corroborating the results obtained by iTRAQ and regular Western blotting analyses. The primary full-length blots can also be found in Supplemental Appendix 3.
involved in the ubiquitination of huntingtin that is mutated in the neurodegenerative disorder Huntington disease (19). Correspondingly, the ubiquitin-proteasome system is linked to monogenic forms of PD (2). Scherzer et al (20) recently identified huntingtin interacting protein 2 as a molecular marker of early PD based on gene expression in blood. For GSTM3, we found that it was decreased in the PD groups in all 3 subcellular fractions (Supplemental Appendix 1, 2; Table). Glutathione S-transferase Mu 3 belongs to the GST family that consists of detoxification enzymes that can counter oxidative and chemical stresses. Glutathione S-transferase Mu 3 has been implied to have potential functions in Alzheimer disease (21). Previous case control studies of GST polymorphisms and PD have also suggested that GSTM1, another member of the GST Mu subclass, might be linked to PD, but the results are still inconsistent (29, 30).

Some other proteins identified in this study are known to be important in the CNS but have not previously been linked to PD. One example is the protein N-Myc down-stream-regulated gene 2, which may be involved in dendritic cell and neuron differentiation and may also have tumor suppressor activity (25). Another example is the excitatory amino acid transporter 2, which is 1 of the 3 glutamate transporters expressed in human motor cortex and plays an important role in the reuptake of released glutamate, thus terminating its postsynaptic action (22). Given that excitotoxicity is also implicated in PD pathogenesis (1, 2), further analysis of the latter protein may shed more light on PD progression.

Among the proteins we have identified, we selected mortalin for further validation of its identification and quantification among different disease stages with an alternative means. The results confirmed that its protein levels were decreased in the cytosolic fraction from the frontal cortex of PD patients, particularly in the PD-limbic and PD-isocortex groups (Figs. 2, 3). However, because most mortalin is primarily expressed in neurons (10), and there is a progressive neuronal loss in the cortex as PD advances, a decrease in the mortalin protein level in the cortex can be due to several possibilities, including: 1) the number of neurons is decreased; 2) the mortalin content itself in each neuron is decreased; and 3) both the neuron number and the mortalin neuron content are reduced as PD advances. Unfortunately, there currently is no suitable way to resolve these issues. Furthermore, because we only detected a decrease in the cytosolic fraction, it is also possible that the decrease in mortalin level was due to a redistribution of the protein in different subcellular compartments (with or without a slight decrease of the total mortalin synthesis/increase of the protein degradation).

Mortalin is a multifunctional protein that not only functions as a molecular chaperone involved in mitochondrial import and energy generation but also as a guardian against oxidative stress that has multiple binding partners (26, 27, 30–33). Furthermore, mortalin may have detrimental effects on the cell due to its proliferative support to cancer cells (10, 26, 33). Several studies have assigned mortalin to multiple subcellular sites such as mitochondria, endoplasmic reticulum, plasma membrane, cytoplasmic vesicles, and cytosol, as examined by a variety of protocols, including immunolectron microscopy (34–41). We demonstrated in a recent study that mortalin expression was decreased in the mitochondrial fraction isolated from the SNpc of PD patients as compared with controls (10). In addition, we demonstrated that mortalin interacts with both α-synuclein and DJ-1 in a DAergic cell line (27). However, the present report is the first that indicates that the expression of mortalin decreased in a quantitative manner in the cytosolic fraction isolated from the frontal cortex of PD patients at different stages. It remains to be determined whether the decrease in mortalin protein levels is the consequence of the disease development/progression or whether mortalin is responsible for the development or progression of PD. In a previous study, we demonstrated that overexpression of mortalin rendered DAergic cells more vulnerable to rotenone-induced neurotoxicity, whereas downregulation of mortalin expression attenuated these effects (10). We also observed that the role of mortalin in DAergic neurodegeneration is associated with oxidative stress as well as mitochondrial and proteasomal function (10), all of which have been implicated in the pathogenesis of PD (1, 2). Although the precise mechanism by which mortalin produces its effects is still unknown, 1 hypothesis is that it acts via DJ-1, mutations of which cause familial PD (2). Consistent with our observations, Li et al reported that DJ-1 was associated with mortalin in addition to heat shock protein 70 and carboxy terminal of heat shock protein 70-interacting protein, and was translocated to mitochondria upon oxidative stress (42). Thus, it is reasonable to propose that mortalin might accomplish its effects by interacting either directly or indirectly with DJ-1 and/or α-synuclein. In view of mortalin’s multiple binding partners (26, 27, 30–33), its diverse cellular functions (26), a role in the survival of DAergic neurons, and its presence in mitochondria and extramitochondrial sites (26, 38), it is likely that changes in levels of mortalin in cortical neurons may also be critical in PD progression.

In conclusion, our report of proteomic profiling by the iTRAQ method provides, for the first time, a novel way to study the mechanisms of PD progression. With pathologically verified human tissues at different disease stages, we identified many novel proteins that likely contribute to the progression of the disease. We validated 1 of these proteins, mortalin, and demonstrated that it was preferentially decreased in the cytosolic fractions. Further biologic studies need to be conducted to elucidate its roles and mode of actions in progression of PD.

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