Proteomic Identification of a Stress Protein, Mortalin/mthsp70/GRP75

RELEVANCE TO PARKINSON DISEASE

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Functional impairment of mitochondria and proteasomes and increased oxidative damage comprise the main pathological phenotypes of Parkinson disease (PD). Using an unbiased quantitative proteomic approach, we compared nigral mitochondrial proteins of PD patients with those from age-matched controls. 119 of 842 identified proteins displayed significant differences in their relative abundance (increase/decrease) between the two groups. We confirmed that one of these, mortalin (mthsp70/GRP75, a mitochondrial stress protein), is substantially decreased in PD brains as well as in a cellular model of PD. In addition, nine candidate mortalin-binding partners were identified as potential mediators of PD pathology. Manipulations of mortalin level in dopaminergic neurons resulted in significant changes in sensitivity to PD phenotypes via pathways involving mitochondrial and proteasomal function as well as oxidative stress. *Molecular & Cellular Proteomics* 5: 1193–1204, 2006.

Parkinson disease (PD) is characterized by preferential dopaminergic (DAergic) neurodegeneration in the substantia nigra pars compacta (SNpc) with subsequent DA loss in the nigrostriatal pathway and the presence of Lewy bodies in the remaining nigral neurons (1). Although the mechanisms underlying PD development remain elusive in both genetic and sporadic PD, mitochondrial and proteasomal dysfunction and oxidative stress are recognized as major contributors (2, 3).

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The pivotal roles of these pathways are further substantiated by the fact that all chemically induced parkinsonian models, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (4), rotenone (5), and more recently epoxomicin (6), lead to mitochondrial and proteasomal dysfunction as well as increased oxidative stress. Nonetheless despite decades of research, identification of molecules involved in these processes in the setting of DAergic neurodegeneration has yielded limited success. Consequently current clinical treatment of PD is at a standstill with the replacement of DA or with DAergic agonistic approaches (7, 8).

In this study, we used an unbiased state-of-the-art proteomic technique called shotgun proteomics multidimensional protein identification technology (MudPIT) to quantitatively profile mitochondrial proteins from pathologically verified PD patients and normal age-matched controls as well as in a cellular model of PD, i.e. DAergic cells treated with parkinsonian toxicant rotenone. MudPIT uses multidimensional LC and tandem mass spectrometry to separate and fragment peptides for protein identification (9) as well as for quantification when used in combination with ICAT and stable isotope labeling by amino acids in cell culture (SILAC) techniques (10, 11). With these approaches, we identified many novel proteins with quantitative expression differences in the SNpc of PD patients as compared with controls. One of these proteins, mortalin/mthsp70/GRP75, decreased significantly in many PD brain samples and in the cellular model of PD. Several mortalin-binding proteins likely participating in rotenone-mediated toxicity were also identified. Furthermore overexpression and silencing of mortalin expression in the cellular model of PD significantly influenced PD type pathologies. Thus, we report for the first time that a mitochondrial stress protein, mortalin, manipulates PD pathogenesis by its mitochondrial and proteasomal functions as well as its effect on oxidative stress.

EXPERIMENTAL PROCEDURES

Human Brain Samples and Immunohistochemistry

Five pathologically verified PD and five healthy control subjects were obtained from Duke University, the University of California at Los Angeles, and the University of Washington. All subjects were age-, gender-, and postmortem interval (<12 h)-matched. The Human Subjects Division from each of the universities approved this study. The neuropathological diagnosis of PD and normal controls were based
on established criteria that are routinely used in our laboratory (12, 13). To perform immunohistochemistry in a separate set of control and PD patients (six PD patients and four controls, all of which were archived cases at the University of Washington), 8-μm-thick sections of nigral tissue were cut from formalin-fixed, paraffin-embedded blocks; all sections were deparaffinized with xylene followed by hydration through graded ethanol and then processed with a standard immunohistochemistry protocol (14). Sections were incubated overnight at 4 °C with mouse anti-GRP75 antibody (1:200, Stressgen Biotechnologies, Victoria, British Columbia, Canada) followed by incubation with biotin-conjugated goat anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA) for half an hour and then revealed by a standard peroxidase method with VIP chromogen (Vector Laboratories) and counterstained with hematoxylin and eosin.

**Sample Preparation before Proteomics**

The SNpc was dissected from controls and PD patients; an equal amount of tissue from each subject was pooled into two samples (i.e. control and PD SNpc) followed by isolation of mitochondria-enriched fractions using a method described previously (15, 16) with minor modifications. Briefly, nigral tissues (~100 mg) were suspended in 1 ml of sucrose buffer containing 20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM PMSF, protease inhibitor mixture (Sigma), and phosphatase inhibitors (0.2 mM Na3VO4 and 1 mM NaF). The suspension was homogenized with 10 strokes using a glass homogenizer and then centrifuged at 4 °C at 800 × g for 10 min to sediment nuclei and unsuspended material. The supernatant was further centrifuged at 10,000 × g for 15 min to obtain the mitochondria-enriched fraction, which was resuspended in a sample buffer consisting of 6 M urea, 10,000 g unsuspended material. The supernatant was further centrifuged at 65–85% B for 5 min. The solvents used for the reversed-phase separation were 0.1% SDS, 5 mM EDTA, and 50 mM Tris-HCl (pH 8.5). Protein concentrations were determined by Bradford assay.

**ICAT Analysis of Mitochondria-enriched Fraction with LTQ MS**

A comparison of the relative abundance of protein profiles in the SNpc of control and PD patients was achieved with the ICAT labeling technique that was initially described by Gygi et al. (17) and is routinely utilized in our laboratory (11, 16, 18, 19). To perform the ICAT experiment, 100 μg of mitochondria-enriched proteins from either PD or control samples were reduced, and the cysteine groups were biotinylated with a 5-fold molar excess of either heavy (13C) (PD) or light (12C) (control) cleavable ICAT reagents. Next the two labeled samples were mixed and digested with trypsin (Promega, Madison, WI) overnight at 37 °C. Then the digested peptide solution was passed consecutively over an ionic exchange column and a monomeric avidin column (Applied Biosystem, Foster City, CA). The biotinylated peptides were eluted with 0.3% TFA in 30% acetonitrile, and biotin was cleaved from the labeled peptides with concentrated TFA. Finally the peptides were separated and analyzed on an LTQ work station (Thermo Electron Corp., San Jose, CA) using automated, on-line two-dimensional LC separation (strong cation exchange and then C18 column) followed by nanoelectrospray LC/MS (20). The eluted peptides from the strong cation exchange column (10–800 mM NH4Cl) were loaded onto one of the peptide traps, while peptides on the other trap and the PicoFrit column (5–μm BioBasic C18: 300-Å pore size; 75 μm × 10 cm; tip, 15 μm; New Objective, Woburn, MA) were eluted with a 0–65% mobile phase B gradient for 60 min and then 65–85% B for 5 min. The solvents used for the reversed-phase column were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) solutions. The solvent for the sample pump was 0.1% formic acid in water (C). A flow rate of 75 μL/min before the split and 250 nl/min after the split were used for the MS pump, and a flow rate of 150 μL/min before splitting and 2 μL/min after splitting were used for the sample pump. The spray voltage was 1.8 kV, the capillary temperature was 150 °C, and 35 units of collision energy were used to obtain the fragment spectra. Two MS/MS spectra of the most intense peaks were obtained following each full-scan mass spectrum. The dynamic exclusion feature was enabled to obtain MS/MS spectra on co-eluting peptides. Proteins from the mixture were later identified automatically using the computer program Sequest™, which searched the MS/MS spectra against the human International Protein Index (IPI, Version 2.33, 43,175 entries) database (11, 16, 18, 19).

**Cellular Model of PD**

The DAergic neurons used in this study are MES cells, which express most features of human DAergic neurons and have been widely used in PD-related experiments (24–26). Detailed methods for culturing MES cells have been previously described by us (26). Cells were seeded overnight and then treated with 2.5–10 nm rotenone (a potent inhibitor of mitochondrial complex I) or vehicle (0.1% DMSO) for 3 days.

**SILAC Analysis of Proteins Associated with Mortalin**

MES cells were grown in culture medium containing [12C]-Arg or [13C]-Arg, respectively, for at least 3 days followed by treatment with 5 nm rotenone (prelabeled with [13C]-Arg or DMSO (prelabeled with [12C]-Arg) for 3 days. The cells were counted and lysed in ice-cold

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Gene Manipulation in MES Cells

Transfection of Mortalin-2 (GRP75) in MES Cells and Selection by Flow Cytometry—GFP-tagged mortalin was expressed from pEGFP-C1/mot-2. Cells were transfected with pEGFP-C1/mot-2 or pEGFP-C1 as described previously (28) and according to the manufacturer’s specifications (FuGENE 6, Roche Applied Science). Clones with high mortalin-2 expression levels were selected by flow cytometry using GFP and replated to form stable cell lines. Mortalin-2 expression levels were assessed by Western blot.

Mortalin siRNA Transfection in MES Cells—MES cells plated in 24-well culture dishes were transfected with 5 μg mouse mortalin-specific siRNA (Mm_Hspa8a_1 HP siRNA (Qiagen, Valencia, CA); the target sequence is CAGGTTTCTGCCAAAGATAAA, located in the middle of the gene (GenBank accession number NM_010481)) constructs or nonspecific control siRNA constructs (Qiagen) using HiPerFect transfection reagent (Qiagen). Twenty-four hours after siRNA transfection, cells were treated with vehicle or 2.5–10 nM rotenone for 3 days. Neurotoxicity was measured by trypan blue exclusion assay, and then the harvested cells were used for Western blot analysis for assessment of mortalin level.

Cell Viability and Functional Analysis

Viability—Untransfected cells and cells transfected with vector or mortalin-2 were seeded in 24-well plates at 25,000 cells/well and treated with vehicle or rotenone at 2.5, 5, and 10 nM, respectively, for 3 days. The cells were collected and mixed with trypan blue dye solution before being counted with a hemacytometer.

Measurement of Mitochondrial Complex I Activity—Mitochondria were isolated and assayed for complex I activity using methods described previously (29). Briefly complex I (NADH-ubiquinone oxidoreductase) activity was measured by monitoring the loss of absorbance at 340 nm (ε = 6.81 mM⁻¹ cm⁻¹) resulting from the oxidation of n-decylubiquinone (130 μM) at 30 °C.

Measurement of Proteasomal Activity—20 S chymotrypsin-like and postglutamyl peptidase proteasomal activities were determined as described previously by measuring the fluorescence of 7-aminomethylcoumarin liberated from peptide-7-amido-4-methylcoumarin-linked substrates (30). The results, expressed as fluorescence units/min/mg of protein, were normalized against inhibition of total proteasomal function by 10 μM lactacystin.

Protein Carbonyl Assay for Oxidative Stress—Protein oxidation was measured by quantifying total protein carbonyl contents using 2,4-dinitrophenylhydrazine (DNPH). The spectrum difference between DNPH-treated and control samples was determined, and results were expressed as nanomoles of DNPH incorporated/mg of protein based on the absorption at 375 nm (ε = 21.0 mM⁻¹ cm⁻¹). Protein concentration was determined by standard BCA assay.

Protein expression levels were assessed by Western blot using antibodies specific to mortalin (Santa Cruz Biotechnology, Santa Cruz, CA), and then protein concentrations were measured by standard BCA assay. Equal amounts of the protein from rotenone- and DMSO-treated cells were loaded and subjected to SDS-PAGE and transferred to nitrocellulose membranes, which were then incubated with mortalin-specific antibodies and then probed with horseradish peroxidase-conjugated secondary antibodies and visualized using chemiluminescence.

Immunofluorescent Staining of Transfected MES Cells and Confocal Microscopy Analysis

MES cells transfected with GFP or GFP-mortalin were seeded on chambered glass slides (Nalge Nunc, Naperville, IL) and then fixed in 4% paraformaldehyde followed by overnight incubation with primary antibody cytochrome c (1:200, BD Pharmingen) and then incubation with secondary antibody (1:200 Flex Fluor® 568 goat anti-mouse IgG, Molecular Probes, Eugene, OR). A laser scanning confocal microscope (Bio-Rad LS2000) was used to capture images.

Western Blot

10 μg of protein from mitochondria-enriched fractions as well as the cytosol-enriched fractions from human SNpc or MES cells, were subjected to 8–16% SDS-PAGE and transferred to PVDF, blocked, and probed overnight at 4 °C with mouse anti-GRP75 antibody (1:20,000, Stressgen Biotechnologies); peroxidase-conjugated secondary antibody was added at 1:2,000 and developed with enhanced chemiluminescence.

Statistical Methods

Grouped data are expressed as mean ± S.E. Changes between groups were analyzed by one-way analysis of variance or Student’s t test using GraphPad Prism 3.0 (San Diego, CA). All measurements were repeated at least three times in all experiments. p < 0.05 was accepted as significant.

RESULTS

Quantitative Analysis of Protein Profiles in the Mitochondria-enriched Fractions—Mitochondria-enriched fractions were pooled from the SNpc of five PD patients and age-matched controls, respectively, using our recently described methods (16). Next three independent ICAT analyses were performed in pooled samples with the identification results of each of the three independent runs combined, yielding identification of a grand total of 842 proteins. Proteins identified with ≥2 peptides and a single peptide are listed in Supplemental Appendix I (a total of 653 proteins/groups) and Supplemental Appendix II (a total of 189 proteins/groups), respectively. The rationales for pooling samples and combining results from all runs are discussed briefly under “Experimental Procedures” but in greater detail in our previous publications (10, 11, 16, 18, 19). As expected, the majority of proteins listed in Supplemental Appendices I and II with known functions are related to mitochondria with a significant portion of them (~40%) being linked to either signal transduction, the ubiquitin proteasomal system, or regulation of oxidative stress, all of which have been implicated in PD pathogenesis (31–33).

Table I lists the 119 proteins that displayed significant changes in relative abundance between PD and controls in at least two independent experiments; these proteins are more likely to play major roles in PD development or progression. For example, a subunit of NADH-ubiquinone oxidoreductase (part of mitochondrial complex I) was significantly decreased in PD compared with controls; this is consistent with earlier observations showing decreased mitochondrial complex I activity in PD patients (34–37). To catalog our data for further

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The results are expressed as \(^{12}\text{C} / {^{13}\text{C}}\) (control) / \(^{13}\text{C} / {^{13}\text{C}}\) (PD). Proteins sharing the same peptides but with different protein identification numbers are listed in one cell.

| No. | Protein name | Mean ± S.E. |
|-----|--------------|-------------|
| ASAP Ratio ≤ 0.5 (increase ≥2-fold in PD) | | |
| 1 | 60 S ribosomal protein L3 (IPI00295392, IPI00219335, IPI00176675) | 0.24 ± 0.02 |
| 2 | 6-Phosphofructokinase (IPI00220617, IPI00332371) | 0.26 ± 0.03 |
| 3 | Aldo-keto reductase family 1, member A (IPI00220271) | 0a |
| 4 | ARL-6-interacting protein-1 (IPI00014232) | 0.24 ± 0.02 |
| 5 | Astrocytic phosphoprotein PEA-15 (IPI00014850) | 0.41 ± 0.19 |
| 6 | Creatine kinase, ubiquitous mitochondrial precursor (IPI00024638) | 0.50 ± 0.03 |
| 7 | Elongation factor 1- (IPI00023048, IPI00305969, IPI00396660, IPI00159917, IPI00164838, IPI00064086, IPI00332119, IPI00100137, IPI00178440) | 0.37 ± 0.05 |
| 8 | Ferrochelatase, mitochondrial precursor (IPI00027776) | 0a |
| 9 | Gelsolin precursor (IPI00026314, IPI00377087) | 0.26 ± 0.12 |
| 10 | Hypothetical protein (IPI00000581) | 0.39 ± 0.11 |
| 11 | Hypothetical protein (IPI00156689) | 0.27 ± 0.01 |
| 12 | Hypothetical protein (IPI00179218) | 0.48 ± 0.04 |
| 13 | Ig \(\mu\) chain C region membrane-bound segment (IPI00382937, IPI00101462, IPI00335356, IPI00385264, IPI00386236) | 0.07 ± 0.01 |
| 14 | Monoglycercide lipase (IPI00293590) | 0a |
| 15 | Protein-tyrosine phosphatase, non-receptor type 5 (IPI0002328575, IPI00383550, IPI00008837) | 0.44 ± 0.05 |
| 16 | Radixin (IPI00173673, IPI00219365) | 0.18 ± 0.27 |
| 17 | Ras-related protein Rab-14 (IPI00291928) | 0.46 ± 0.03 |
| 18 | Ras-related protein Rab-21 (IPI00007755) | 0.36 ± 0.19 |
| 19 | Similar to 60-kDa heat shock protein, mitochondrial precursor (hsp60) (IPI00179377, IPI00239351, IPI00234131, IPI00176823) | 0.30 ± 0.13 |
| 20 | Splice isoform 1 of Q9UI40 sodium/potassium/calcium exchanger 2 precursor (IPI00006444, IPI00218809) | 0.30 ± 0.07 |
| 21 | Splice isoform DM-20 of P06905 myelin proteolipid protein (IPI00022776) | 0.19 ± 0.41 |
| 22 | Synaptoporin (IPI00029184) | 0.39 ± 0.16 |
| 23 | Transmembrane protein 10 (IPI00006444) | 0.47 ± 0.04 |
| 24 | Tubulin \(\alpha\)-chain (IPI00007755) | 0.10 ± 0.01 |
| 25 | Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial precursor (IPI00182020, IPI00026964) | 0.19 ± 0.01 |
| 0.5 < ASAP Ratio ≤ 0.75 (increased between 1.25- and 2-fold in PD) | | |
| 26 | Adenylate kinase isoenzyme 1 (IPI00018342) | 0.67 ± 0.19 |
| 27 | \(\alpha\)-Actinin 4 (IPI00013808) | 0.75 ± 0.05 |
| 28 | ATP-dependent helicase DDX1 (IPI00293655) | 0.54 ± 0.17 |
| 29 | Brain link protein-1 precursor (IPI00291809) | 0.56 ± 0.10 |
| 30 | Breast carcinoma amplified sequence 1 (IPI0002328575) | 0.57 ± 0.03 |
| 31 | Carbonyl reductase 1 (IPI00293586) | 0.69 ± 0.13 |
| 32 | General vesicular transport factor p115 (IPI00031583) | 0.51 ± 0.04 |
| 33 | Glutathione S-transferase M2 (IPI00219067) | 0.53 ± 0.03 |
| 34 | Heat shock 20-kDa-like protein p20 (IPI00022433) | 0.57 ± 0.03 |
| 35 | Histidine triad nucleotide-binding protein 1 (IPI00239077) | 0.72 ± 0.10 |
| 36 | Hypothetical protein (IPI00016381) | 0.62 ± 0.09 |
| 37 | Hypothetical protein (IPI00000581) | 0.61 ± 0.04 |
| 38 | Integrin \(\beta\)8 precursor (IPI00029533) | 0.59 ± 0.05 |
| 39 | Nitilase homolog 1 (IPI00023779) | 0.53 ± 0.13 |
| 40 | Nuclear transport factor 2 (IPI00009901) | 0.64 ± 0.05 |
| 41 | Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (IPI0013723) | 0.66 ± 0.11 |
| 42 | Peroxiredoxin 1 (IPI00008837) | 0.55 ± 0.05 |
| 43 | Protein-tyrosine phosphatase, non-receptor type 5 (IPI0002328575, IPI00383550, IPI00008837) | 0.44 ± 0.05 |
| 44 | Rab GDP dissociation inhibitor \(\alpha\) (IPI00010154) | 0.53 ± 0.03 |
| 45 | | |
| 46 | | |

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**Mortalin and Parkinson Disease**

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### Table I—continued

| No. | Protein name | Mean ± S.E. |
|-----|--------------|-------------|
| 47  | Rab GDP dissociation inhibitor β (IPI00031461) | 0.67 ± 0.03 |
| 48  | Ras-related protein Rab-3C (IPI00061114, IPI00023504) | 0.54 ± 0.02 |
| 49  | Ras-related protein Rap-1b (IPI00015148, IPI00032808, IPI00019345, IPI00387007) | 0.64 ± 0.06 |
| 50  | Rho-related GTP-binding protein RhoG (IPI00017342) | 0.54 ± 0.03 |
| 51  | Secretogranin I precursor (Sgl) (IPI00006601) | 0.60 ± 0.04 |
| 52  | Septin 7 (IPI00033025) | 0.58 ± 0.15 |
| 53  | Septin KIAA0202d (IPI00333841, IPI00022082) | 0.72 ± 0.06 |
| 54  | SH3-containing protein SH3GLB2 (IPI00024540) | 0.59 ± 0.03 |
| 55  | Similar to glycolipid transfer protein (GLTP) (IPI00260424, IPI00184363) | 0.51 ± 0.03 |
| 56  | Sodium/hydrogen exchanger 1 | 0.51 ± 0.03 |
| 57  | Sorcin (IPI00396248, IPI00027175) | 0.73 ± 0.07 |
| 58  | Splice isoform 1 of O14775 guanine nucleotide-binding protein β subunit 5 (IPI00151607, IPI00022065, IPI00007787) | 0.72 ± 0.16 |
| 59  | Splice isoform 2 of Q8IYJ6 NAD-dependent deacetylase sirtuin 2 (IPI00382551, IPI00032853, IPI00032852, IPI00179109) | 0.58 ± 0.15 |
| 60  | Splice isoform β-1D of P05556 integrin β1 precursor (IPI00217563, IPI00009465, IPI00217561, IPI00217562, IPI00107540, IPI00293005) | 0.60 ± 0.04 |
| 61  | Splice isoform CNPI of P09543 2; 3′-cyclic nucleotide 3′-phosphodiesterase | 0.56 ± 0.13 |
| 62  | Splice isoform IC2 of O00499 Myc box-dependent interacting protein 1 (IPI00329733, IPI00395680, IPI00220587, IPI00220997, IPI00221000, IPI00186966, IPI00221001, IPI00220999, IPI00220586, IPI00220998, IPI00220996) | 0.61 ± 0.08 |
| 63  | Splice isoform SERCA1B of O14983 sarcoplasmic/endoplasmic reticulum calcium ATPase 1 | 0.74 ± 0.05 |
| 64  | Transforming protein RhoB (IPI00000041) | 0.61 ± 0.14 |
| 65  | Tubulin β-2 chain (IPI00007752, IPI00160897, IPI00023598) | 0.71 ± 0.09 |

**ASAP Ratio ≥ 2 (decreased ≥2-fold in PD)**

| No. | Protein name | Mean ± S.E. |
|-----|--------------|-------------|
| 66  | 14-3-3 protein ε (IPI00000816, IPI00086909, IPI00092176) | 2.19 ± 0.38 |
| 67  | 3′,5′-Cyclic nucleotide phosphodiesterase 10A2 (IPI00297440, IPI00215786) | 2.38 ± 0.17 |
| 68  | Acylphosphatase 2 (IPI00216461) | 3.24 ± 0.17 |
| 69  | Aldehyde dehydrogenase 1A1 (IPI00218914) | 2.60 ± 0.24 |
| 70  | α-Centactin, β-centactin (IPI00029468, IPI00029469) | 3.13 ± 0.32 |
| 71  | C-terminal binding protein 1 (IPI00385665, IPI00012835, IPI00390642) | 2.59 ± 0.29 |
| 72  | Diaphorase-related protein-1 (IPI00184899) | 2.37 ± 0.15 |
| 73  | Dynamin-like protein (IPI00235412, IPI00146935, IPI00037283) | 12.66 ± 0.88 |
| 74  | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial precursor (IPI00032875) | 2.72 ± 0.17 |
| 75  | Endonuclease dicer (IPI00219036) | 3.34 ± 0.19 |
| 76  | Glutamate decarboxylase, 67-kDa isoform (IPI00292646) | 999a |
| 77  | Glutathione transferase Omega 1 (IPI00019755) | 2.83 ± 0.15 |
| 78  | Guanine nucleotide-binding protein G/Gαs subunit (IPI00003058) | 2.01 ± 0.45 |
| 79  | Guanine nucleotide-binding protein, α11β1subunit (IPI00290928) | 999a |
| 80  | Hypothetical protein (IPI00395655, IPI00154742, IPI00061246, IPI00382938, IPI00395435, IPI00395412, IPI00386785, IPI00382876, IPI00386158, IPI00396153, IPI00384931, IPI00384355) | 2.86 ± 0.76 |
| 81  | Hypothetical protein FLJ30394 (IPI00289396) | 5.63 ± 0.51 |
| 82  | Methylmalonate-semialdehyde dehydrogenase (acylating), mitochondrial precursor b (IPI00024990) | 3.84 ± 0.30 |
| 83  | NADH-ubiquinone oxidoreductase 42-kDa subunit, mitochondrial precursor (IPI00029561) | 2.92 ± 0.50 |
| 84  | NADH-ubiquinone oxidoreductase subunit B14.7 (IPI00329301) | 3.60 ± 0.26 |
| 85  | Protein transport protein Sec23A (IPI00017375) | 999a |
| 86  | Pyruvate dehydrogenase protein X component, mitochondrial precursor (IPI00298423) | 4.89 ± 1.40 |
| 87  | Similar to adaptor-related protein complex 2, β 1 subunit (IPI00098756) | 2.60 ± 0.20 |
| 88  | Similar to ribosomal protein L18a (IPI00233081, IPI00176629, IPI00375890, IPI00026202) | 3.42 ± 0.34 |
| 89  | Splice isoform 1 of O14168 MAGUK p55 subfamily member 2 (IPI00217808, IPI00218271) | 3.44 ± 0.21 |
| 90  | Stress-70 protein, mitochondrial precursor (mortalin) (IPI00007765) | 2.19 ± 0.68 |
| 91  | Tumor protein D53 (IPI00383670) | 2.40 ± 0.15 |
analysis, we have separated the proteins into four groups in alphabetical order within each category (Table I); proteins whose levels 1) increased ≥2-fold (ASAP Ratio ≤ 0.5), 2) increased between 1.25- and 2-fold (0.5 < ASAP Ratio ≤ 0.75), 3) decreased ≥2-fold (ASAP Ratio ≥ 2), and 4) decreased between 1.25- and 2-fold (1.25 ≤ ASAP Ratio < 2) when 100 µg of protein from PD was paired with 100 µg from control.

Validation of Mortalin with Western Blot Analysis in PD Patients—The current protein database is incomplete; hence proteins inferred from peptide sequence could be wrong. Consequently candidate proteins identified by proteomics need to be validated before their biological roles are pursued extensively. In our first step toward verifying proteins most likely to have biological importance, we chose mortalin (also referred to as stress-70 protein, mitochondrial precursor (mths70), PBP74, or GRP75), whose relative level in mitochondria-enriched fractions decreased significantly in PD compared with controls. Biological testing of this molecule in other systems indicates its critical role in cell proliferation and survival (38). As shown in Fig. 1A, the expression of mortalin was not only detected by an alternative method, Western blot analysis, in both cytosol- and mitochondria-enriched fractions but also appeared to be less abundant in mitochondria-enriched fractions of PD samples compared with controls. Semi-quantitative analysis of bands indicated that the mortalin level was decreased by about 2-fold in PD, consistent with our proteomic analysis findings.

To test whether mortalin was actually expressed in the remaining DAergic neurons in PD patients, immunohistochemical studies with mortalin antibody were carried out in a separate set of PD patients (n = 6) and controls (n = 4). Results demonstrated that, although glial staining was minimal, mortalin expression was readily visible in both DAergic and non-DAergic neurons (not shown). Furthermore it appeared that the expression level in controls (Fig. 1B, left panel) was stronger than in PD patients (Fig. 1B, right panel), i.e. consistent with proteomic analysis. Finally mortalin staining

| No. | Protein name | Mean ± S.E. |
|-----|--------------|-------------|
| 92  | Acetolactate synthase (IPI00386719, IPI00009963) | 1.82 ± 0.17 |
| 93  | α-Intelexin (IPI00001453) | 1.72 ± 0.18 |
| 94  | β aducin (IPI00220241, IPI00220240, IPI0019904) | 1.47 ± 0.11 |
| 95  | Calcium/calcmodulin-dependent 3,5'-cyclic nucleotide phosphodiesterase 1B (IPI00005592) | 1.91 ± 0.33 |
| 96  | Calcium/calcmodulin-dependent protein kinase IIB (IPI00377174, IPI00182944, IPI00334271, IPI00219166, IPI00183066, IPI00221305, IPI00235789) | 1.42 ± 0.29 |
| 97  | Cysteine desulfurase, mitochondrial precursor (IPI00295240) | 1.43 ± 0.11 |
| 98  | Cytochrome c oxidase polypeptide I (IPI00016864) | 1.57 ± 0.13 |
| 99  | Elongation factor 2 (IPI00186290) | 0.75), 2) and 4) decreased between 1.25- and 2-fold (1.25 ≤ ASAP Ratio < 2) when 100 µg of protein from PD was paired with 100 µg from control.

**TABLE I—continued**

| No. | Protein name | Mean ± S.E. |
|-----|--------------|-------------|
| 100 | Eukaryotic initiation factor 5A variant A (IPI00376005, IPI00395322, IPI00218084, IPI00006935) | 1.92 ± 0.08 |
| 101 | Excitatory amino acid transporter 1 (IPI00015473) | 1.46 ± 0.08 |
| 102 | Glutamate decarboxylase, 65-kDa isoform (IPI0012796) | 1.91 ± 0.38 |
| 103 | Glutamate dehydrogenase 1, mitochondrial precursor (IPI00016801) | 1.25 ± 0.17 |
| 104 | Glutathione transferase Omega 1 (IPI0019755) | 1.60 ± 0.15 |
| 105 | Guanine nucleotide-binding protein G/G3/G3 subunit (IPI00244567) | 1.63 ± 0.08 |
| 106 | Hypothetical protein FLJ34988 (IPI00176221) | 1.34 ± 0.09 |
| 107 | Hypothetical protein KIAA0821 (IPI00183445) | 1.88 ± 0.13 |
| 108 | Megakaryocyte-stimulating factor (IPI00024825) | 1.30 ± 0.05 |
| 109 | Neurocan core protein precursor (IPI00159927) | 1.42 ± 0.11 |
| 110 | OX-2 membrane glycoprotein precursor (IPI00013162, IPI00221361) | 1.39 ± 0.11 |
| 111 | Peptidyl-prolyl cis-trans isomerase, mitochondrial precursor (IPI00026519) | 1.57 ± 0.11 |
| 112 | Plexin-B1/SEP receptor precursor (IPI00006644, IPI00304929) | 1.85 ± 0.13 |
| 113 | Pyruvate kinase 3 isoform 2 (IPI00220644) | 1.83 ± 0.31 |
| 114 | Ras-related protein Rab-10 (IPI00016513) | 1.40 ± 0.11 |
| 115 | Sodium/potassium-transporting ATPase β-2 chain (IPI00293971) | 1.49 ± 0.07 |
| 116 | Succinate-semialdehyde dehydrogenase, mitochondrial precursor (IPI00336008, IPI0019888) | 1.39 ± 0.10 |
| 117 | Synaptophysin (IPI00027770) | 1.40 ± 0.16 |
| 118 | Telomerase-binding protein p23 (IPI00015029) | 1.45 ± 0.15 |
| 119 | Vesicular inhibitory amino acid transporter (IPI00026015) | 1.47 ± 0.10 |
was absent in Lewy bodies (Fig. 1B, right panel, inset).

Both proteomic and Western blot analyses were done with pooled samples; this approach, although advantageous in many aspects, cannot determine whether the decrease in mortalin resulted from a large decrease in a single patient or from smaller decreases in multiple patients. Furthermore although immunohistochemical studies were performed in individual cases, it is not a reliable technique for demonstrating decreased mortalin expression in DAergic neurons of PD patients compared with controls (Fig. 1B). To address these issues, Western blot analyses were conducted in individual cases from which the samples were pooled. Fig. 1, C and D, show that mortalin expression decreased in the majority of mitochondria-enriched fractions from the PD cases (p < 0.05), whereas there was no significant change in cytosol-enriched fractions.

Investigation of Mortalin in a Cellular Model of PD—Rotenone, a potent mitochondrial inhibitor, produces selective DAergic neurodegeneration with formation of Lewy body-like inclusions in the remaining DAergic neurons in rodents (5, 39). We as well as others have demonstrated in a cellular model of PD that rotenone-mediated neurotoxicity is largely associated with increased oxidative stress and/or proteasomal dysfunction (5, 40). Thus, we next investigated whether a decrease in mortalin level is upheld in the cellular model of PD. As can be seen in Fig. 2A, consistent with our previous results (11), rotenone exposure at 2.5–10 nM produced a dose-dependent neurotoxicity 3 days after treatment. More importantly, although rotenone exposure had no significant effect on mortalin level when total lysate was analyzed, mortalin level was reduced in the mitochondria-enriched fractions (Fig. 2B), consistent with the results obtained in human SNpc as shown in Fig. 1.

Given that the mortalin level decreased preferentially in the mitochondria in both PD and a cellular model of PD, we used a novel proteomic technique (SILAC) (41), which was recently established in our laboratory (10), to investigate proteins associated with mortalin with an emphasis on mitochondria-related proteins. The major advantage of this technique, which is only applicable to cells in culture, is that it labels cells before experiments start, thereby removing most false positives due to processing inconsistency or variations in labeling efficiency that could occur in ICAT assays. In this study, prelabeled MES cells ([12C]Arg versus [13C]Arg) were treated with vehicle or rotenone at 5 nM for 3 days, and then an equal amount of proteins were combined from control and treated cell lysates before mortalin and associated proteins were immunoprecipitated and identified by MS. This analysis re-
Cells were seeded in 100-mm dishes at 3.08E+4 cells/cm² and treated with rotenone. Induced toxicity was assessed using trypan blue exclusion assay and expressed as percentage of vehicle-treated controls. Mortalin and Parkinson Disease

Among the mortalin-associated proteins that are related to mitochondria, some, e.g. thymidine kinase 2 (mitochondrial precursor), were regulated by rotenone, whereas others, e.g. hsp60, were not. Although changes in the relative abundance of the mortalin-associated protein complex after rotenone treatment (samples prepared via immunoprecipitation) cannot be directly compared with those in the human tissue analysis (samples prepared via fractionation), several similarities and differences were observed between these two analyses. For instance, although acyl-CoA dehydrogenase (short/branched chain-specific) showed no change in either human or cellular PD models, the relative abundance of hsp60 was increased by more than 100% in human PD samples (Table I), yet its relative abundance did not change in the mortalin-associated protein complex after rotenone exposure.

**Effects of Manipulating Mortalin Expression on Rotenone-induced Neurotoxicity**—Investigation of mortalin in human PD and a cellular model of PD have clearly indicated that mortalin and/or its associated proteins are likely to play major roles in PD pathogenesis. Thus, we went on to ask whether the loss of mortalin was contributing to toxicity and whether its replacement would offset such toxicity. Fig. 3A, left panel, shows that transfection of the mortalin gene increased mortalin expression to 200% of controls transfected with empty vector. To study the cellular distribution of overexpressed mortalin, we analyzed GFP-tagged mortalin in both fractionated cells and its relation to the mitochondrial protein cytochrome c. It is clear that not only did mortalin transfection increase cellular mortalin, but the mortalin level was also increased preferentially in the mitochondrial fraction (Fig. 3B). Semiquantitative analysis indicated that the mortalin level after gene transfection was 2- and 1.5-fold of vector-transfected cells in the mitochondria- and cytosol-enriched fractions, respectively. In addition, the majority of GFP-tagged mortalin was co-localized with cytochrome c in the mitochondria, whereas control GFP was distributed throughout the cells (Fig. 3C), further confirming that overexpressed mortalin was indeed transported into the mitochondria. When cell viability was measured (Fig. 3D), mortalin overexpression had no effect on cell viability in vehicle-treated cells (Fig. 3D, left panel) but substantially enhanced rather than attenuated rotenone-mediated neurotoxicity (Fig. 3D, right panel), suggesting that rotenone toxicity may be mediated in part via mortalin or its associated proteins with the eventual outcome being reduced mitochondrial mortalin level.

Next we tested the effects of silencing mortalin. As dem-

**Table II**

| Mortalin-associated proteins |
|-----------------------------|
| Not all mortalin-associated proteins were identified in human tissue analysis (Supplemental Appendices I and II), and one of the major reasons could be variation in MS sampling. ↓ decreased more than 2-fold; ↑, decreased between 1.25- and 2-fold; ↑↑, increased more than 2-fold. |

| Proteins with significant changes in relative abundance after rotenone exposure |
|-----------------------------|
| Acyl-CoA dehydrogenase family member 9, mitochondrial precursor |
| Phosphate carrier protein, mitochondrial precursor |
| Thymidine kinase 2, mitochondrial precursor |

| Proteins with no significant changes in relative abundance after rotenone exposure |
|-----------------------------|
| 60-kDa heat shock protein, mitochondrial precursor |
| Acyl-CoA dehydrogenase, short/branched chain-specific, mitochondrial precursor |
| Heat shock protein 75 kDa, mitochondrial precursor |
| NAD(P) transhydrogenase, mitochondrial precursor |
| Similar to ATPase, H⁺-transporting, V1 subunit A, isoform 1 |
| Similar to mitochondrial ribosomal protein S27 |

**Fig. 2. Cell viability assay and mortalin expression in MES cells treated with rotenone.** A, rotenone-induced neurotoxicity in MES cells. Cells were seeded in 100-mm dishes at 3.08E+4 cells/cm² and treated with vehicle or 2.5–10 nM rotenone for 3 days. Rotenone-induced toxicity was assessed using trypan blue exclusion assay and expressed as percentage of vehicle-treated controls. B, Western blot analysis of mortalin in MES cells treated with rotenone. MES cells were fractionated into cytosol (cyt) - and mitochondria (mt)-enriched fractions after treatment with DMSO or rotenone at 5 nM for 3 days. Relative expression levels of mortalin were determined by calculating the total OD for each fraction (representing equal starting material). Although the overall mortalin level was not different between rotenone- versus DMSO-treated cells, its level was preferentially decreased in the mitochondria-enriched fraction in PD compared with controls (`, p < 0.05).
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Fig. 3. Effects of manipulating the mortalin gene in DAergic neurons. A, mortalin expression level in MES cells transfected with expression vector or siRNA. MES cells were transfected with empty plasmid versus plasmid containing GFP-mortalin (left panel) or nonspecific (NC)-siRNA versus mot-2-siRNA (right panel), respectively. Cells were lysed at 48 h after transfection, and expression levels of mortalin were analyzed by Western blot. Each lane contained 10 µg of protein. Molecular masses of mortalin and GFP-mortalin are 74 and 100 kDa, respectively. B, Western blot analysis of mortalin in mitochondria- and cytosol-enriched fractions. Cells transfected with vector (GFP) or mortalin (Mot-2) were lysed and fractionated into cytosol (cyt-) and mitochondria (mt)-enriched fractions. The mortalin expression level was analyzed by Western blot. Each lane contained 10 µg of protein. C, co-localization of GFP-mortalin and cytochrome c in mitochondria. Cells transfected with vector (Row 1, labeled as MES-pEGFP) or mortalin (Row 2, labeled as MES-pmot-2) were fixed in 4% paraformaldehyde followed by overnight incubation with primary antibody cytochrome c and then incubation with Flex Fluor 568 goat anti-mouse secondary antibody. A laser scanning confocal microscope was used to capture images, demonstrating that most of the mortalin (green color in Column 1) was co-localized with cytochrome c (red color in Column 2), i.e. shown in Column 3 as yellow color after merging green and red channels. D, increasing and decreasing mortalin expression had no effect on viability in control cells but significantly influenced rotenone-induced neurotoxicity. Untransfected cells or cells transfected with expression vector or siRNA were seeded in 100-mm dishes at 3.08E4 cells/cm² and treated with vehicle or 5 nM rotenone for 3 days. Rotenone-induced toxicity was assessed using trypan blue exclusion assay and expressed as percentage of controls. *, p < 0.05 for cells transfected with mortalin or mot-2-siRNA compared with vehicle transfection; **, p < 0.01 for cells transfected with mortalin or mot-2-siRNA compared with vehicle transfection.

Tests demonstrated in Fig. 3A, right panel, mortalin level decreased to less than 10% of controls 2 days after gene silencing was initiated. Similar to the results obtained in the overexpression study, reducing mortalin level was nontoxic to MES cells treated with vehicles (Fig. 3D, left panel). Consistent with our results seen with overexpression, decreasing native mortalin protein level protected MES cells from rotenone-mediated neurotoxicity (Fig. 3D, right panel), strongly suggesting mortalin is an essential component of the rotenone effect.

Effects of Mortalin Transfection on Mitochondrial Complex I Activity, Oxidative Stress, and Proteasomal Function—Although the above experiments argue solidly that mortalin is a major target of rotenone, the mechanisms involved are largely unknown. It is well known that rotenone is a potent inhibitor of mitochondrial complex I that in turn produces significant oxidative stress and inhibits proteasomal function in MES cells (5, 42). To further determine the role(s) of mortalin in the rotenone effect, we compared the effects of rotenone on mitochondrial complex I and proteasomal function as well as oxidative stress in cells with or without overexpression of mortalin. Similar to our observation that overexpression of mortalin was nontoxic in the absence of rotenone (see Fig. 3D), we found no difference in these endpoints between untreated cells with and without overexpressed mortalin. However, in the presence of rotenone, we observed marked differences. Consistent with the observations of others (5, 42) as well as our own (11), 5 nM rotenone treatment of untransfected and vector-transfected cells for 3 days significantly suppressed mitochondrial complex I activity, increased oxidative stress, and induced inhibition of both chymotrypsin-like and glutamyl peptidase activities. Also consistent with the observation of neurotoxicity shown in Fig. 3D, mortalin transfection...
A significantly enhanced mitochondrial inhibition, oxidative stress, and proteasomal dysfunction induced by rotenone (Fig. 4).

**DISCUSSION**

Our current study yielded several major findings. First, 842 proteins were identified in the mitochondria-enriched fractions isolated from human SNpc; of these, 119 proteins displayed significant differences in their relative abundance between PD patients and controls. One of the validated proteins, mortalin, although not associated with Lewy bodies, was expressed in DAergic neurons. Second, as in human PD, mortalin also preferentially decreased in the mitochondrial fraction of DAergic cells treated with rotenone, and a few candidate proteins likely participating in rotenone-mediated toxicity were identified. Third, overexpression and silencing of mortalin in DAergic cells significantly influenced cell viability only when cells were treated with rotenone, suggesting that mortalin is one of the major targets of rotenone. Finally, mortalin influenced rotenone-mediated toxicity via pathways involving oxidative stress and mitochondrial and proteasomal dysfunction.

A total of 842 proteins were identified and quantified with a robust proteomic technique in the mitochondria-enriched fractions isolated from human SNpc; however, some mitochondrial proteins reported in the literature were not observed in our study. Besides the limitation of MS, a few other potential explanations can account for this discrepancy, including the following. 1) Some mitochondrial proteins do not contain cysteine residues (e.g. cytochrome oxidase polypeptide Vic) and thus are transparent to the ICAT method, and 2) several reported mitochondrial proteins such as mitochondrial import inner membrane translocase subunit TIM8 B, were identified in our study by one peptide but failed to meet our criteria or those of most investigators in proteomic research (43). Finally, it should be noted that a few cytoplasmic proteins are also listed in Supplemental Appendices I and II; this should not be surprising as the samples used were mitochondria-enriched fractions, not purified mitochondria, and thus contained a few other membranous elements.

ICAT quantification of the mitochondria-enriched fractions of SNpc identified numerous proteins with significant quantitative differences between PD and controls. In particular, numerous subunits of the mitochondrial complexes were substantially decreased in PD patients, including those associated with complex I (e.g. NADH-ubiquinone 42-kDa B14.7 subunit) as well as other mitochondrial complexes including cytochrome c oxidase (complex III) polypeptide I. These observations are noteworthy as they provide protein substrates for mitochondrial dysfunction in PD pathogenesis (16). Nonetheless despite the fact that all proteins with quantitative differences between two groups may have a potential role in PD pathogenesis, all of them need to be validated not only for identification but also quantification (as we did for mortalin) before their functional roles are pursued extensively. This is because proteins could be misidentified by proteomics due to the incompleteness of the current database.

SILAC analysis of mortalin-associated proteins after rotenone exposure revealed many proteins, some of which are clearly related to mitochondrial function. It is obvious that proteins whose levels are modulated by rotenone, e.g. acyl-CoA dehydrogenase family member 9 and phosphate carrier protein (Table II), are worth further pursuit because they are likely to be key players in mediating the effects of mortalin on rotenone-induced neurotoxicity. On the other hand, despite the fact that rotenone did not change the level of hsp60 in...
mortalin-associated protein complex, it is still possible that the function of hsp60 could be altered if the nature of mortalins changes (see “Discussion” below). It should be noted here that hsp60 has already been validated recently by one of our co-authors to interact with mortalins both in vivo and in vitro in a different experimental system (44).

In the current study, we validated that the mortalin level was not only decreased in the mitochondrial fraction of PD patients but also in a cellular model of PD. Previous studies, largely focused on cancer biology, have suggested a role of mortalin as an antiapoptosis protein because 1) overexpression of mortalins resulted in malignant transformation of NIH 3T3 (45) and lifespan extension of normal human fibroblasts (46), 2) expression levels of mortalin were elevated in human brain tumors (47), and 3) reduction in the mortalin level by its antisense expression caused senescence-like growth arrest in immortalized human cells (48). The function(s) of mortalins in DAergic cells, or more specifically in the mitochondria of these cells, is unknown. Neither overexpression nor silencing of mortalin level had any significant effects on the viability of MES cells treated with vehicle, suggesting that decreasing mortalin level alone is probably insufficient in causing neurodegeneration. In fact, a decrease in mortalin level has also been observed in aging kidneys (49). Nonetheless, overexpression of mortalins rendered DAergic cells more vulnerable to rotenone-induced neurotoxicity, whereas down-regulated mortalins expression produced opposite effects, arguing strongly that mortalin is a critical player in rotenone-mediated neurotoxicity. We ruled out the possibility that increased neurotoxicity was due to the inability of overexpressed mortalins to enter the mitochondria, thereby disrupting normal cytosolic protein functions (Fig. 3). We also confirmed that the mechanisms by which mortalins mediate rotenone associated toxicity involved enhanced oxidative stress as well as mitochondrial and proteasomal dysfunction (Fig. 4), all of which have been implicated in PD pathogenesis. The question that remains to be answered is how precisely does mortalin execute its effect on rotenone-mediated neurotoxicity? Rotenone exposure reduced the mitochondrial mortalin level, but silencing native mortalins offset rotenone-mediated toxicity, suggesting that it is the delicate balance of mortalins and its associated proteins (not necessarily the absolute level of mortalins) that determines the sensitivity of DAergic cells to rotenone. To this end, we have already identified several mortalins-binding proteins, and their biological functions in rotenone-mediated toxicity will be investigated further. Finally, mortalins appears to be oxidized in an animal model of Alzheimer disease (50), raising the possibility that changes in mortalin structure may also be important in neurodegenerative diseases. It is feasible that increased oxidative stress due to rotenone exposure oxidizes mortalins, particularly when it is overexpressed, thereby influencing the functions of mortalins and/or its associated proteins, e.g., hsp60, and ultimately affecting rotenone-induced neurotoxicity. However, it should be noted that we have not examined the nature and/or extent of post-translational modification of mortalins, including whether it is oxidized when exposed to rotenone, in our studies. Post-translational modification of mortalins, if present in this model, can be as important as the change in its relative abundance in terms of modulating rotenone-mediated neurotoxicity or even human PD.

In summary, with pathologically verified human tissues we identified numerous novel proteins that likely contribute to PD pathogenesis. We validated one of these proteins, mortalins, in both human samples and a cellular model of PD and demonstrated that it was preferentially decreased in mitochondrial fractions. Furthermore overexpression and silencing of mortalin level in DAergic cells significantly influenced cell viability when treated with rotenone, suggesting that mortalins is a major mediator of neurotoxicity induced by this widely used parkinsonian toxicant. Finally although detailed mechanisms underlying the interactions between mortalins and its associated proteins remain to be characterized, the effects of mortalins on rotenone-mediated toxicity appeared to involve oxidative stress as well as mitochondrial and proteasomal dysfunction.

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