Novel Monoclonal Antibodies Demonstrate Biochemical Variation of Brain Parkin with Age*

Received for publication, June 27, 2003, and in revised form, September 3, 2003
Published, JBC Papers in Press, September 12, 2003, DOI 10.1074/jbc.M300889200

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Autosomal recessive juvenile parkinsonism is a movement disorder associated with the degeneration of dopaminergic neurons in substantia nigra pars compacta. The loss of functional parkin caused by parkin gene mutations is the most common single cause of juvenile parkinsonism. Parkin has been shown to aid in protecting cells from endoplasmic reticulum and oxidative stressors presumably due to ubiquitin ligase activity of parkin that targets proteins for proteasomal degradation. However, studies on parkin have been impeded because of limited reagents specific for this protein. Here we report the generation and characterization of a panel of parkin-specific monoclonal antibodies. Biochemical analyses indicate that parkin is present only in the high salt-extractable fraction of mouse brain, whereas it is present in both the high salt-extractable and RIPA-resistant, SDS-extractable fraction in young human brain. Parkin is present at decreased levels in the high salt-extractable fraction and at increased levels in the SDS-extractable fraction from aged human brain. This shift in the extractability of parkin upon aging is seen in humans but not in mice, demonstrating species-specific differences in the biochemical characteristics of murine versus human parkin. Finally, by using these highly specific anti-parkin monoclonal antibodies, it was not possible to detect parkin in α-synuclein-enriched preparations from human brain. Thus, challenging prior inferences about the role of parkin in movement disorders other than autosomal recessive juvenile parkinsonism.

Autosomal recessive juvenile parkinsonism (AR-JP)† is an especially insidious form of parkinsonism that can strike as early as the 1st decade of life. A major locus for this disease was mapped to chromosome 6q, and the gene was subsequently identified and termed parkin (1). Human parkin is a 465-amino acid protein with a predicted molecular mass of 52 kDa that contains an N-terminal ubiquitin-like domain, a linker region, and a C-terminal TRIAD domain consisting of two RING fingers on either side of an in-between RING (IBR) finger region (2). Deletions and insertions of one or more exons resulting in premature translation termination are some of the most common mutations in parkin, but numerous missense point mutations in parkin have also been shown to be causal of AR-JP (3). Since the identification of parkin, many studies focused on elucidating the function of this protein, and it has been shown it can function as a ubiquitin-protein isopeptide ligase and that its overexpression in vivo enhances the ubiquitinylation of synphilin-1, Pael-R, and CDCRel-1. Immunoprecipitated parkin has been reported to catalyze the ubiquitinylation of these substrates in vitro as well as O-glycosylated α-synuclein p22 and cyclin E (4–8). Furthermore, mutations linked to AR-JP have been reported to reduce the ubiquitinylation of these substrates. Moreover, emerging data suggest that parkin may protect cells from premature death by targeting misfolded or damaged proteins for degradation via the ubiquitin proteasome pathway (4, 18, 19, 38).

Numerous studies (5, 8–22) have reported that parkin is extractable from cultured cells as well as from human and rodent brain by using low salt buffers with or without the addition of mild detergents. Parkin has also been found in Lewy body-enriched preparations from human brain (20). However, there is a discrepancy among these reports on the number and size of human versus rodent parkin isoforms as well as discrepancies in the subcellular distribution of parkin (12, 13, 15, 21, 23). To clarify some of these issues, sensitive and specific antibodies to parkin were generated. A panel of anti-parkin monoclonal antibodies (mAb) that recognize different domains of parkin was produced, and their specificity in murine and human brain was demonstrated. Detailed analyses of the biochemical properties of parkin in mouse and human brain tissue by using serial fractionation with buffers of increasing protein extraction strength showed unusual properties of parkin in the human brain. Finally, by using these highly specific parkin mAbs in conjunction with biochemical and immunostaining methods, we were unable to detect parkin in α-synuclein containing lesions in patients with α-synucleinopa-

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* This work was supported in part by NIA and NINDS grants from the National Institutes of Health and the Michael J. Fox Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to this work.

‡ Supported by a National Institutes of Health training grant (Training in Age-related Neurodegenerative Diseases).

¶ Supported by a fellowship from the Canadian Institutes of Health Research.

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§§ The abbreviations used are: AR-JP, autosomal recessive juvenile parkinsonism; ELISA, enzyme-linked immunosorbent assay; DLB, dementia with Lewy bodies; GCI, glial cytoplasmic inclusion; HS, high salt buffer; HST, high salt Triton buffer; LS, low salt buffer; mAb, monoclonal antibody; MSA, multiple system atrophy; PBS, phosphate-buffered saline; PD, Parkinson’s disease; PMI, post-mortem interval; PMSF, phenylmethylsulfonyl fluoride; RIPA, radioactive immunoprecipitation assay buffer; IBR, in-between RING finger; CHO, Chinese hamster ovary; DAPI, 4',6-diamidino-2-phenylindole.
Biochemical Detection of Parkin in Human and Mouse

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Human Parkin

The full-length human parkin cDNA was cloned into the baculovirus expression vector pFASTBAC-HTb and expressed in Sf9 cells (Invitrogen). The resulting His6-tagged parkin protein was purified on a column packed with nickel-nitrilotriacetic acid resin (Invitrogen). In brief, Sf9/parkin cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.5, 0.1 M KCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 1% Nonidet P-40 and then centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was applied to a nickel-nitrilotriacetic acid column pre-equilibrated in equilibration buffer (20 mM Tris-HCl, pH 8.5, 0.5 M KCl, 20 mM imidazole, 5 mM 2-mercaptoethanol, 1 mM PMSF, 10% glycerol). The column was washed with 10 column volumes of the same buffer followed by 2 column volumes of wash buffer (20 mM Tris-HCl, pH 8.5, 1.5 M KCl, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 10% glycerol) and then 2 column volumes of equilibration buffer. His6-parkin was eluted with 20 mM Tris-HCl, pH 8.5, 0.1 M KCl, 100 mM imidazole, 5 mM 2-mercaptoethanol, 1 mM PMSF, and 10% glycerol (see Fig. 1A).

Antibody Generation, Epitope Mapping, and Isootyping

The rabbit polyclonal antibodies CS2132 and AB5112 were purchased from Cell Signaling Technology (Beverly, MA) and Chemicon International, Inc. (Temecula, CA), respectively. Murine anti-human parkin mAbs were generated by immunization of mice with recombinant human parkin as described (30). Fusion was conducted by using spleen lymphocytes from immunized Balb/c mice and SP2 cells to produce hybridomas. Resulting hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) using plates coated with parkin. The epitopes of the anti-parkin mAbs were mapped by Western blotting using proteins expressed by Gene-Porter 2 (Gene Therapy Systems, San Diego, CA)-mediated transfection of QB293 cells with pcDNA3.1-myc/His (Invitrogen) harboring subcloned domains of parkin. To generate these constructs, PCR fragments of QBI293 cells with pcDNA3.1-myc/His (Invitrogen) harboring subcloned domains of parkin. To generate these constructs, PCR fragments spanning the coding region for each domain (ubiquitin-like domain, 1–77; linker region, 78–220; first RING finger, 221–305; IIB region, 306–398; second RING finger, 399–465) were digested and ligated into the host vector. The predicted sequence of each construct was confirmed by DNA sequencing. Antibodies were isolated by antigen-captured ELISA by using goat anti-mouse antibodies to each immunoglobulin subtype (Sigma).

Gel Electrophoresis and Western Blotting

Proteins were resolved on 10% and 15% SDS-polyacrylamide gels for parkin and α-synuclein, respectively, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) as described previously (29, 30). Western blotting was conducted by following published protocols by using either goat anti-mouse (Jackson Immuno-Research Laboratories, West Grove, PA) or goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to horseradish peroxidase as secondary antibodies (29, 30). Immuno-positive protein bands were detected with Renaissance Enhanced Lumino Reagents (PerkinElmer Life Sciences) and X-Omat Blue XB-1 film (Eastman Kodak Co.).

Cell Culture and Generation of Cell Lysates

Wild type Chinese hamster ovary (CHO) cells and CHO cells stably expressing human parkin (CHO-PAR4) were maintained in a minimal essential medium (Invitrogen), 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 units/ml streptomycin. HeLa, Neuro-2A, and HEK-293 cells were maintained in Dulbecco’s modification of Eagle’s medium (Cellgro by Mediatech, Herndon, VA), 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. HeLa, Neuro-2A, and HEK-293 cells were maintained in Dulbecco’s modification of Eagle’s medium (Cellgro by Mediatech, Herndon, VA), 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. HeLa, Neuro-2A, and HEK-293 cells were maintained in Dulbecco’s modification of Eagle’s medium (Cellgro by Mediatech, Herndon, VA), 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were grown to ~80% confluence, lysed in 2% SDS, 50 mM Tris, pH 7.6, and heated to 100 °C for 5 min. Protein concentrations of cell lysates were determined by bichoninic acid assay (Pierce) prior to SDS-PAGE and Western blotting.

Tissue and Sequential Biochemical Fractionation

Parkin-null mice were generated by genomic deletion of exon 2 of parkin, removing most of the coding region for the ubiquitin-like do-

thies thereby calling into question prior studies about the role of parkin in movement disorders other than AR-JP.

Table I

Summary of subjects used in this study

| Case    | Diagnosis | Age | Sex | PMI |
|---------|-----------|-----|-----|-----|
| Y-1     | NML       | 14  | M   | 10  |
| Y-2     | NML       | 23  | M   | 8   |
| Y-3     | NML       | 22  | M   | 4   |
| A-1     | SCHZ      | 86  | F   | 7.5 |
| A-2     | NML       | 92  | F   | 5   |
| A-3     | SCHZ      | 59  | F   | 7.5 |
| A-4     | SCHZ      | 70  | F   | 17.5|
| A-5     | NML       | 69  | M   | 11  |
| A-6     | SCHZ      | 76  | F   | 10  |
| A-7     | NML       | 60  | M   | 14  |
| A-8     | NML       | 74  | F   | 6   |
| A-9     | NML       | 74  | F   | 3.5 |
| A-10    | NML       | 43  | M   | 30.5|
| A-11    | DLB       | 90  | F   | 5   |
| A-12    | DLB       | 75  | F   | 6   |
| A-13    | DLB       | 79  | M   | 20.5|
| A-14    | NML       | 75  | M   | 17  |
| A-15    | MSA       | 65  | M   | 43  |
| A-16    | MSA       | 57  | F   | 8   |
| A-17    | MSA       | 54  | M   | 25  |

The parkin-null mice were maintained on a C57Bl/6 × Sv129S hybrid background. Young wild type and parkin null mice used in these studies were 4–12 weeks old, and aged wild type mice were >22 months old. Whole mouse brains were dissected for biochemical fractionation studies. The brain tissue samples from human subjects that were used for biochemical analyses are summarized in Table I. Two extraction methods (1 and II) were used for these tissues as summarized below.

Method I—Murine or human tissue samples were homogenized in 2 ml/g of high salt (HS) buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 750 mM NaCl) and sedimented at 125,000 × g for 30 min at 4 °C. Supernatants from the initial fractionation were saved as the HS fraction, and the pellets were washed and re-extracted sequentially with buffers of increasing protein extraction strengths. For each buffer, two cycles of extraction and washes were conducted using 2 ml/g of HS + 1% Triton X-100 (HST) followed by 2 ml/g of RIPA (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris, pH 8.0). Supernatants were saved as the HST and RIPA fractions, respectively. Floatation and removal of contaminating myelin using HS + 20% sucrose was performed prior to the RIPA extraction. A final extraction using 2 ml/g of SDS, 50 mM Tris, pH 7.6, was conducted on the pellet and sedimented at 125,000 × g for 30 min at 22 °C. The supernatant was saved as the SDS fraction.

Method II—Brain tissues from patients with α-synucleinopathies and control patients were homogenized in 10 ml/g low salt (LS) buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol, and 10% sucrose) and sedimented at 25,000 × g for 30 min at 4 °C. Supernatants were saved as the LS fraction, and pellets were washed by re-extraction in LS buffer. Resulting pellets were subjected to two sequential extractions in 10 ml/g Triton X-100 (TX) buffer (LS + 1% Triton X-100 + 0.5 mM NaCl) and sedimented at 180,000 × g for 30 min at 4 °C. Supernatants from the first of these extractions were saved as the TX fraction. Pellets were then homogenized in 10 ml/g Sarkosyl buffer (LS + 1% N-lauroyl sarcosine + 0.5 mM NaCl) and incubated at 22 °C on a shaker for 1 h prior to sedimentation at 180,000 × g for 30 min at 22 °C. Supernatants were saved as the Sarkosyl extraction buffer fraction. Remaining pellets were extracted in 2.5 ml/g SDS buffer (2% SDS, 50 mM Tris, pH 7.6) prior to centrifugation at 25,000 × g for 30 min at 22 °C. Supernatants were saved as the SDS fraction.

All extraction buffers contained a mixture of protease inhibitors (1 μg/ml each of leupeptin, pepstatin A, N-p-tosyl-1-phenylalanyl chloromethyl ketone, N-p-tosyl-L-lysine chloromethyl ketone, soybean trypsin inhibitor, and 0.5 mM PMSF). For experiments using quantitative amounts of protein, total protein concentration was determined with the BCA protein assay kit (Pierce) using bovine serum albumin as

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a standard. SDS sample buffer (10 mM Tris, pH 6.8, 1 mM EDTA, 40 mM dithiothreitol, 1% SDS, 10% sucrose) was added to samples of HS, HST, RIPA, LS, TX, and Sarkosyl extraction buffer fractions; sample buffer without SDS was added to the SDS samples. All samples were heated for 5 min at 100 °C prior to SDS-PAGE.

**Immunopurification of Parkin from Human Brain**

PRK8-coupled dextran beads were generated using the Carbo-Link column kit (Pierce) following the manufacturer’s protocol. Proteins were extracted from human frontal cortex tissue by two extractions with HST, and myelin was floated and removed as described above. The resulting pellet was homogenized in 8M urea in 50 mM Tris, pH 7.6, with protease inhibitors. Following sedimentation at 125,000 × g, the supernatant was diluted to 2 mM urea and incubated by gentle rocking with PRK8-coupled beads for 4 h. The incubation and all subsequent steps were performed at 4 °C. The beads were washed 3 times with 2 mM urea, 1% Triton X-100 in 50 mM Tris, pH 7.6, and bound parkin was then eluted with Pierce ImmunoElution Buffer by gentle rocking for 1 h. SDS sample buffer was added to an aliquot of the eluant. Samples were not boiled to limit carbamoylation of protein in the presence of urea.

**Immunofluorescence and Immunohistochemistry**

The harvesting, fixation, and further processing of the tissue specimens used in this study were conducted as described previously (24, 25). Briefly, tissue blocks of cingulate cortex from DLB or cerebellum from MSA brains were fixed with 70% ethanol, 150 mM NaCl or neutral buffered formalin and infiltrated with paraffin. The diagnostic assessment of all DLB and MSA cases (both of which are α-synucleinopathies characterized by α-synuclein inclusions) was performed in accordance with published guidelines (26, 37). Whole mouse brains (wild type or parkin null) were fixed with 70% ethanol, 150 mM NaCl and paraffin-embedded.

Immunohistochemistry was carried out using the avidin-biotin complex detection system (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine as described (24, 25). The anti-synuclein mouse monoclonal antibody Syn303 (28) was used to detect α-synuclein inclusions by immunohistochemistry as described previously (27–30). Sections were also lightly counter-stained with hematoxylin.

In order to try to stain α-synuclein pathological inclusions in human diseased cases with parkin antibodies, a variety of fixation and retrieval methods were applied. These include the following: 1) formalin-
These four parkin mAbs detected proteins in extracts from parkin null mice (Fig. 1 and Table II). While at the same time showing no cross-reactivity with pro-

PRK101 Md + – IgG1
PRK106 R2 ++ + + IgG1
PRK107 R2 ++ ++ IgG1
PRK109 R2 ++ ++ ++ IgG1
CS2132 R2 + – Polyclonal
Ab5112 ND + – Polyclonal

Characterization of selected anti-parkin antibodies

| Antibody | Domaina | Humanb | Mousec | Isotype |
|----------|---------|--------|--------|---------|
| PRK3     | R2      | + –    | –      | IgG2a   |
| PRK5     | R2      | + + +  | + + +  | IgG2b   |
| PRK28    | R2      | + + +  | + + +  | IgG1    |
| PRK35    | UbL     | + –    | –      | IgM     |
| PRK36    | IBR     | + –    | –      | IgG1    |
| PRK101   | Md      | + –    | –      | IgG1    |
| PRK106   | R2      | ++ +   | +      | IgG1    |
| PRK107   | R2      | ++     | +      | IgG1    |
| PRK109   | R2      | ++ +   | ++ +   | IgG1    |
| CS2132   | R2      | + –    | –      | Polyclonal |
| Ab5112   | ND      | + –    | –      | Polyclonal |

α UbL, ubiquitin-like domain; Md, linker domain; IBR, in-between RINGs domain; R2, second RING domain; ND, not determined.

1 Ability of antibody to detect an immunoreactive protein band(s) that may correspond to human parkin in the 2% SDS fraction of human brain extract: +, weak; + +, moderate; + + +, strong.

2 Ability of antibody to detect a band corresponding to parkin in the high salt fraction of wild-type mouse brain which is absent in the parkin-null mouse brain: -, unable to detect specific band; +, weak specific band; + +, moderate specific band; + + +, strong specific band.

TABLE II

biochemical distribution of parkin in mouse and human brain. Western blot analysis of sequential extracts (method I; see “Experimental Procedures”) from wild type (A) and parkin-null mouse brain (B) using the antibody PRK8. Immunoblot analysis with antibody PRK8 of HS- or HST- (C) and RIPA- or SDS (D)-soluble extracts generated by method I from five cortical regions of cases A-1 (upper panels) and A-2 (lower panels). 40 μg of total protein were loaded per lane for HS and HST fractions, and 7.5 μg of total protein were loaded per lane for RIPA and SDS fractions. The mobility of molecular mass markers (kDa) is shown on the left of each panel. rec. 2 ng of recombinant tagged human parkin. cing. cingulate, frontal, temporal, occipital and parietal cortices, respectively. E, immunoprecipitation (IP) followed by Western blotting (WB) analysis to demonstrate that the RIPA-resistant, SDS-soluble immunoband is parkin. RIPA-insoluble human brain lysate was immunoprecipitated with PRK8, and Western blot analysis was performed using the indicated anti-parkin mAbs. One-quarter adjusted volume of the starting material (S) was loaded relative to the amount of immunoprecipitated material (IP) to account for the incomplete pull-down of parkin.

Double labeling indirect immunofluorescence analyses for brain sections were performed as described previously (29). The rabbit anti-

Experimental Procedures.

To produce antibodies specific to parkin, a battery of murine mAbs was raised against recombinant human parkin as described under “Experimental Procedures.” We identified 60 hybridomas with strong immunoreactivity for the recombinant protein by ELISA. To confirm the specificity of these parkin mAbs, we conducted Western blot analyses using brain extracts from wild type and parkin-null mice as well as extracts from the human brain. Surprisingly, out of our panel of 60 mAbs, only four anti-parkin antibodies (PRK8, PRK28, PRK106, and PRK109) were able to detect protein bands with apparent molecular masses corresponding to that of parkin while at the same time showing no cross-reactivity with proteins in extracts from parkin null mice (Fig. 1 and Table II). These four parkin mAbs detected ~50- and ~46-kDa bands in mouse brain extracts but not in extracts from parkin-null mice; representative Western blots are shown in Fig. 1B. A similar doublet of ~50- and ~46-kDa immunobands was also detected in human brain extracts with PRK8, PRK28, PRK35, PRK106, PRK109, and a commercially available antibody, CS2132 (Figs. 1B and 2). A very minor cross-reacting band at ~142 kDa was detected in both wild type and parkin-null mouse brain extracts by some of the parkin mAbs, but this ~142-kDa band is the major species recognized by CS2132 (Fig. 1B). The other commercially purchased anti-parkin antibody AB5112 did not recognize authentic parkin since this antibody detected a number of protein bands in mouse brain extracts that were also present in brain extracts from parkin-null mice (Fig. 1B).

To identify specific domains within parkin that are recognized by our mAbs, we conducted immunoblot analysis using lysates generated from QBI293 cells overexpressing each of the Myc/His-tagged parkin domains (Fig. 1, C and D, and Table II). The majority of the mAbs appeared to bind to the RING2 domain at the C terminus, whereas two of the mAbs detected the ubiquitin-like domain with a single mAb detecting the IBR domain and the region between amino acid residues 76 and 238 of parkin. Despite the generation of mAbs specific for different
domains of parkin overexpressed in QBI293 cells, only PRK8, PRK28, PRK106, and PRK109 reacted strongly with full-length parkin in brain extracts without evidence of other contaminating protein bands. Thus, only mAbs to the RING2 domain of parkin were suitable for direct in vivo studies. Although it is possible that some antibodies to other domains of parkin (e.g. PRK3, PRK35, PRK36, and PRK101) may be specific for human parkin and they may not cross-react with murine parkin (Table II), it was not possible to unequivocally confirm their specificity in extracts from human brains.

To confirm the ability of anti-parkin mAbs to detect endogenous parkin in cell lines, we performed Western blots by using lysates of CHO cells stably transfected with human parkin (CHO-PAR4) as well as untransfected CHO, HeLa, Neuro2A, SH-SY5Y, and HEK-293 cells. Both PRK8 and PRK28 detected an ~50-kDa band corresponding to endogenous parkin in CHO, SH-SY5Y, and HEK-293 cells, although the levels of parkin in these cell lines were much lower than in the CHO-PAR4 line (Fig. 1E).

To characterize the biochemical properties of parkin, we performed serial extractions on brain tissue from mouse and human using buffers of increasing protein extraction strengths (Fig. 2). In mouse, parkin was extractable in HS buffer alone as shown with antibody PRK8 (Fig. 2A). No parkin was detected in the HST, RIPA, or SDS fractions, indicating that little or no RIPA-resistant parkin was present in the mouse brain. The ~44- and 50-kDa protein bands detected with PRK8 in HS fraction were confirmed to correspond to parkin, because these immunobands are not present in extract from null-parkin mice (Fig. 2B). Similar results were obtained with antibodies PRK28 and PRK109 (data not shown). By contrast, the biochemical properties of parkin in aged human brain differed greatly from that of mouse brain. Little or no human parkin was detected in the HS fraction, and the majority of parkin was found in the RIPA-resistant, SDS-extractable fraction (Fig. 2, C and D). The data shown in Fig. 2, C and D, with antibody PRK8 was confirmed with PRK28, PRK35, and PRK109 (data not shown). Indeed, SDS-extractable parkin was persistently present in the gray matter of cingulate, frontal, temporal, occipital, and parietal cortex from six aged human cases examined here (Fig. 2, C and D, and Table III). Furthermore, in addition to 2% SDS, we found that RIPA-resistant parkin in human brain also can be extracted by buffer containing 8 M urea (data not shown). To verify that the immunoreactive species extracted by either 2% SDS or 8 M urea from human brain was indeed parkin, we immunoprecipitated parkin using PRK8-coupled beads after diluting the 8 M urea fraction to 2 M urea. Immunoprecipitated parkin was detected by parkin mAbs that recognize either the N (PRK35) or C termini (PRK8, PRK28, and PRK109) of parkin (Fig. 2E). The ability of this panel of mAbs to detect PRK8-immunoprecipitated material demonstrated that parkin was indeed present in a highly insoluble form in human brain. Parkin also was variably detectable in the HS and HST fractions in some of the cases examined here. The presence or absence of HS/HST-extractable parkin varied from case to case, and when present, it was typically most highly extractable from temporal cortex (Table III). This analysis was extended to include the caudate, putamen, globus pallidus, and cerebellum from three of the aged human cases. Although the majority of the parkin was recovered in the RIPA-resistant, SDS-extractable fraction from these regions, some HS/HST-extractable parkin was present in cases that exhibit an HS/HST-extractable cortical pool of parkin (data not shown).

It is possible that the differences in the biochemical properties of murine and human parkin are related to age or different postmortem intervals (PMIs). To address these possibilities, we compared serially extracted parkin from brain tissue of young human (14–22 years) and young (4–12 weeks) and aged (>22 months) mice, as well as in tissue from mice with variable PMI. There was no observable difference in the extractability of parkin extracted from young or old mice or as a function of increasing PMI up to 6 h (data not shown). However, the extractability of parkin in the frontal cortex of the three young human cases differed from parkin in the frontal cortex of the elderly human cases in that significant amounts of parkin were recovered from both the HS- and RIPA-resistant, SDS-extract-
able fraction of young cases (Fig. 3A). Interestingly, human parkin was not detected in the RIPA fractions. Despite the presence of higher levels of HS-extractable parkin in brain tissue from young human cases, significant amounts of SDS-extractable material also were present in these cases (Fig. 3, B and C). These results were confirmed with two other parkin antibodies, PRK28 and PRK109 (data not shown). Thus, there is an effect of aging on parkin in human but not in mouse brains.

Because several reports have suggested that parkin and α-synuclein are linked functionally or pathologically (5, 17, 20), we examined the ability of our anti-parkin mAbs to detect a biochemical association between these two proteins as well as evidence of parkin in α-synuclein inclusions of patients with DLB and MSA. First, we showed that LS-extractable α-synuclein, as detected by LB509 (33), is present in similar amounts from cingulate cortical gray matter of DLB and control cases (Fig. 3D). However, parkin levels were consistently low in the LS fraction of both disease and control cases except in one case (A-10) wherein high levels of LS-extractable parkin were most likely due to the relatively young age (43 years) of this individual. As shown previously (24, 33–36), pathological α-synuclein was present in the RIPA-resistant, SDS-extractable fraction from only diseased cases (Fig. 3E), and as expected, parkin was present in the SDS-extractable fraction from both disease and normal cases. The variation in parkin levels in the SDS-extractable fraction could be due to variable cell loss in disease cases. Thus, RIPA-insoluble parkin levels do not strongly correlate with α-synuclein levels in pathological brain tissue.

To explore further the association of parkin with α-synuclein, we asked whether or not parkin could be detected in Lewy bodies and Lewy neurites from patients with DLB or PD, as well as glial cytoplasmic inclusions (GCIs) from patients with MSA. Immunohistochemical studies using parkin mAbs PRK8, PRK28, or PRK109 and anti-α-synuclein antibodies on adjacent brain sections from DLB and MSA cases failed to detect parkin in any α-synuclein inclusions (Fig. 4, A–D; data not shown). Double labeling indirect immunofluorescence studies also revealed no detectable parkin staining in Lewy bodies, Lewy neurites, or GCIs (Fig. 4, E–H). In addition to the methods shown here, a variety of antigen preservation or retrieval methods (see “Experimental Procedures”) was used to try to detect parkin in α-synuclein pathological inclusions. To confirm that our parkin antibodies can detect parkin in fixed cells, immunofluorescence analysis of transfected cells overexpressing parkin was performed. Several parkin mAbs, including

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**Fig. 3.** Biochemical distribution of brain parkin in young and aged human as well as in patients with DLB and MSA. All the fractions generated by method I from case Y-1 (A) and the specific fractions HS (B) and SDS (C) from frontal cortex of three young (Y-1, Y-2, and Y-3) and three aged (A-7, A-8, and A-9) cases were loaded on SDS-polyacrylamide gels, and following electrophoretic transfer onto membranes the blots were probed with PRK8. LS (D) and SDS (E) fractions generated by sequential extraction method II from gray matter of cingulate cortex from DLB cases and cerebellar white matter from MSA cases, and from control cases were analyzed by Western blot analysis and probed with PRK8 (upper panels) and the anti-α-synuclein antibody LB509 (lower panels). The mobility of molecular mass markers (kDa) is shown at the left of each panel. rec, 2 ng of tagged recombinant human parkin.
PRK8, PRK28, and PRK109, specifically and robustly recognized parkin in transfected cells (Fig. 4; data not shown).

Moreover, we also wanted to demonstrate that our novel anti-parkin antibodies could specifically detect parkin in fixed, paraffin-embedded transfected cells and in mouse brain. CHO cells, transfected to stably express human parkin (see “Experimental Procedures”), were generated, and these cells were processed and paraffin-embedded using the standard method used for human tissue. Following sectioning and incubation with several anti-parkin antibodies (i.e. PRK8 and PRK28 (Fig. 5) or PRK109 (data not shown)), cells expressing parkin could readily be detected (Fig. 5). Furthermore, PRK28 was able to detect endogenous parkin in mouse brain by immunohistochemistry. In mouse cerebellum, PRK28 detected parkin in white matter, the molecular layer, and Purkinje cells (Fig. 5M). This staining was not seen in parkin null mice (Fig. 5N), indicating the specificity of this antibody for mouse parkin. PRK28 revealed a similar diffuse staining pattern throughout the brains of wild type mice, whereas parkin null mice showed no staining (data not shown).

**DISCUSSION**

We have generated a library of murine anti-parkin mAbs that detect recombinant parkin by ELISA and immunoblotting. Initial analyses using extracts from human and mouse tissues, however, were variable and difficult to interpret. Attempts at detecting parkin by using published protocols and our mAbs as well as commercially available and published parkin antibodies from other groups (data not shown) resulted in the detection of protein bands that, although close to the predicted size of 52 kDa for parkin, differed subtly in size depending on the antibody used. The difficulty in identifying antibodies specific to parkin is likely borne out by recent analysis of human genomic data, which identified RING fingers as the fourth most abundant protein domain in the human genome, as it is present in 379 predicted human proteins (31). Moreover, the most recent analysis of the human proteome performed by the EMBL-EBI (www.ebi.ac.uk/index.html) indicates that the average protein length in the human proteome is 459 amino acid residues, very close to that of parkin. It is therefore likely that there are many RING finger proteins with a molecular mass close to that of parkin detectable as cross-reacting species by many anti-parkin antibodies generated here as well as by commercial sources and other investigators. Nevertheless, four of our anti-parkin mAbs detected parkin specifically because these mAbs recognized protein bands from human and mouse brains but not in

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**Fig. 4.** Parkin immunofluorescence and immunohistochemistry. Shown are bright field images of a Lewy body in a PD patient stained with Syn303 (A, arrowhead) and lack of staining with PRK8 of the same Lewy body (B, arrowhead). Staining of GCIs in a patient with MSA with Syn303 (C, arrow) and in an adjacent section GCIs are unstained by PRK109 (D). Immunofluorescence staining of α-synuclein inclusions (green) with antibody SNL-4 (E–H) and the lack thereof with parkin antibodies (red) PRK8 (E), PRK28 (F and G), and PRK109 (H) in substantia nigra of DLB cases (E and F) and cerebellum of MSA (G and H). Staining with PRK109 of QBI-293 cells transfected with a parkin expression vector (I). A–D, the nuclei of cells were stained with hematoxylin; E–I, they were visualized with DAPI (blue). Bar, A–H, 50 μm; I, 33 μm.

**Fig. 5.** Parkin immunostaining of paraffin-embedded CHO cells and mouse brain. Untransfected CHO cells (A–C and G–I) or a mass culture of CHO cells stably expressing full-length parkin (D–F and J–L) were fixed with ethanol and paraffin-embedded as described under “Experimental Procedures.” Cells stained with PRK8 (A–F) and PRK28 (G–L) followed by Alexa Fluor 594-conjugated anti-mouse antibody (left column) and counterstained with DAPI (middle column) to visualize the nuclei. The overlays are shown in the right column. Brains from wild type (M) and parkin null (N) mice were fixed with ethanol, paraffin-embedded, and immunohistochemically stained with PRK28 as described under “Experimental Procedures.” PRK28 revealed diffuse staining in the white matter, Purkinje cells, and the molecular layer of wild type mouse cerebellum (M) which was not seen in parkin null mice (N). Bar, A–L, 100 μm; M and N, 67 μm.
Biochemical Detection of Parkin in Human and Mouse

bially expressing parkin as well as endogenous expression of parkin changes with age or in response to chemicals or environmental stressors. The recent finding (6) that parkin is present in Skp1, cullin, and F-box protein-like complexes supports such a possibility.

Whereas several reports (17, 20) have detected parkin in α-synucleinopathies with some anti-parkin antibodies, we were unable to detect parkin in Lewy bodies, Lewy neurites, or GCIIs by immunohistochemical or immunofluorescence techniques. However, we have mAbs that recognize both N- and C-terminal regions of human parkin, and they do not detect any additional human isoforms of parkin.

Interestingly, although mouse parkin was easily extracted from brain by HS buffer, most human parkin was only extracted from brain with harsher buffers, such as 2% SDS, especially in elderly humans. One explanation for this phenomenon is that human parkin becomes modified with age or interacts with other molecules that become modified with age, and these alterations influence extractability. These modifications may not occur in mice either because of differences in mouse proteins or because of the shorter life span of mice. It will be important to determine whether the biological functions of parkin are affected by changes in extractability. If the less readily extracted forms of parkin are compromised in their intrinsic activity or availability to substrates, that would support the observation that haplo-insufficiency might contribute to PD (32). Alternatively, parkin may be part of different protein complexes that have different extraction properties, and the relative abundance of these complexes may change with age. The recent finding (6) that parkin is present in Spk1, cullin, and F-box protein-like complexes supports such a possibility.

Whereas several reports (17, 20) have detected parkin in α-synucleinopathies with some anti-parkin antibodies, we were unable to detect parkin in Lewy bodies, Lewy neurites, or GCIIs by immunohistochemical or immunofluorescence techniques. We were, however, able to detect diffuse cytoplasmic staining of parkin in cells overexpressing parkin. We were also able to detect parkin in ethanol-fixed, paraffin-embedded cells stably expressing parkin as well as at endogenous expression levels in mouse brain, demonstrating that our mAbs are capable of recognizing parkin by using immunohistochemical methods. Because disease-related proteins tend to be quite concentrated in characteristic aggregates and we failed to detect parkin in these α-synuclein containing inclusions, our data argue against an interaction between parkin and α-synuclein in α-synucleinopathies.

The generation of sensitive anti-parkin antibodies, with their specificity confirmed by testing on parkin-null mouse tissues, has provided us with powerful tools for examining the properties of parkin in mouse and human brains. Two isoforms of parkin have been identified in both mouse and human brains, although the precise nature of these two isoforms remains unclear. We have determined that the biochemical properties of parkin are inherently different between mouse and human brains and that there is a shift in parkin from the HS/HST-extractable to RF-resistant fractions with age in the human brain. It is possible that alterations in the biochemical properties of parkin with age are a contributing factor to the development of PD, but further work is necessary to examine the nature and roles of the multiple parkin isoforms and to determine the mechanism(s) whereby the extractability of parkin changes with age.

Acknowledgments—We thank Mark Cookson (National Institutes of Health) for the gift of a plasmid containing the parkin cDNA, Jennifer Norris for maintaining S9 insect cells, and the families of patients who made this research possible.

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