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Parkin and CASK/LIN-2 Associate via a PDZ-mediated Interaction and Are Co-localized in Lipid Rafts and Postsynaptic Densities in Brain*

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Mutations in the gene encoding parkin cause an autosomal recessive juvenile-onset form of Parkinson's disease. Parkin functions as a RING-type E3 ubiquitin-ligase, coordinating the transfer of ubiquitin to substrate proteins and thereby targeting them for degradation by the proteasome. We now report that the extreme C terminus of parkin, which is selectively truncated by a Parkinson's disease-causing mutation, functions as a class II PDZ-binding motif that binds CASK, the mammalian homolog of *Caenorhabditis elegans* Lin-2, but not other PDZ proteins in brain extracts. Importantly, parkin co-localizes with CASK at synapses in cultured cortical neurons as well as in postsynaptic densities and lipid rafts in brain. Further, parkin associates not only with CASK but also with other postsynaptic proteins in the *N*-methyl D-aspartate (NMDA) receptor-signaling complex, in rat brain *in vivo*. Finally, despite exhibiting E2-dependent ubiquitin ligase activity, rat brain parkin does not ubiquitinate CASK, suggesting that CASK may function in targeting or scaffolding parkin within the postsynaptic complex rather than as a direct substrate for parkin-mediated ubiquitination. These data implicate for the first time a PDZ-mediated interaction between parkin and CASK in neurodegeneration and possibly in ubiquitination of proteins involved in synaptic transmission and plasticity.

Parkinson's disease (PD)[1] involves the selective degeneration of midbrain dopamine neurons, resulting in motor abnormalities and progressive disability. Recently, three genes responsible for inherited forms of PD have been identified. Mutations in the genes encoding α-synuclein and ubiquitin C-terminal hydrolase L1 (UCH-L1) each cause rare autosomal dominant forms of PD (1, 2). In contrast, mutations in the gene encoding parkin cause an autosomal recessive, juvenile-onset form of PD and account for more cases of PD than all other familial causes combined (3, 4). Further, Lewy bodies, the cytoplasmic inclusions that constitute the pathological hallmark of the disease, contain α-synuclein, UCH-L1 and parkin deposits even in sporadic cases, suggesting a broader role for these three genes in PD (2, 5, 6). Interestingly however, Lewy bodies do not occur in the brains of patients with familial PD caused by parkin mutations, implicating parkin in the biogenesis of these inclusions (3).

Another major component of Lewy bodies is ubiquitin (Ub), a small protein that can be covalently attached to other proteins (7). Conjugation of Ub onto proteins requires the concerted activity of three enzymes, an E1 Ub-activating enzyme, an E2 Ub-conjugating enzyme, and an E3 Ub-ligase. Ubiquitinated proteins are then targeted for degradation by the 26 S proteasome. The ubiquitin proteasome pathway (UPP) is one of the main pathways for protein degradation and has been implicated in a number of important cellular regulatory processes (8). There is considerable evidence that parkin also functions in the UPP. Indeed, the N terminus of parkin shares homology with ubiquitin (Ub-like domain or Ubl) and its C terminus contains RING domains, a structural feature shared by a large, otherwise divergent, family of E3 Ub-ligases. E3 Ub-ligases are involved in recognizing protein substrates and thereby regulating and conferring specificity to ubiquitination (9). Importantly, several groups have now shown that parkin functions as an E3 Ub-ligase by coordinating the E2-dependent transfer of Ub to target proteins (10, 11). Thus, parkin-mediated ubiquitination and targeting of substrates to the proteasome for degradation could regulate their levels within the cell. Further, defects in parkin-mediated ubiquitination could result in the accumulation of potentially toxic substrates, leading to neurodegeneration.

Identifying the substrates of parkin-mediated ubiquitination may therefore provide insight into the mechanisms of dopamine neuron degeneration in PD. Such critical substrates may be enriched within the same subcellular compartment as parkin. Interestingly, we noticed that the sequence at the extreme C terminus of parkin contains a potential postsynaptic density-95, disc large, zona occludens (PDZ) binding motif, suggesting it could interact with PDZ proteins. PDZ proteins are involved in targeting, clustering, and assembling multiprotein signaling complexes at the postsynaptic density (PSD) (12). PDZ domains contain a conserved peptide-binding groove that interacts with the extreme C terminus of ligands. PDZ domains have been categorized into three classes based on target sequence specificity. Class I PDZ domains bind (S/T)*X*(V/I/L), class II PDZ domains bind -Φ−*X*-Φ (where Φ is a hydrophobic residue) and class III PDZ domains bind (D/E)*X*(V/L) peptide sequences (12). The sequence FDV at the C terminus of parkin corresponds to

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[1] The abbreviations used are: PD, Parkinson's disease; Ub, ubiquitin; GST, glutathione *S*-transferase; SPM, synaptic plasma membrane; SV, synaptic vesicles; NMDA, *N*-methyl D-aspartate; PSD, postsynaptic density; PDZ, postsynaptic density-95, disc large, zona occludens.

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a class II PDZ binding motif. Interestingly, a familial PD-causing mutation, truncating parkin at tryptophan 453, just 12 amino acids from this PDZ binding motif, but leaving the RING domains involved in E3 Ub-ligase activity intact (4, 11), further suggests a critical role for PDZ binding in PD. We report here that parkin is found in a Triton X-100-resistant compartment of the synaptic plasma membrane, similarly to many PDZ-associated proteins. Further, the C terminus of parkin specifically binds to the class II PDZ protein CASK, the mammalian homolog of *Caenorhabditis elegans* Lin-2 (13, 14) but not to other PDZ proteins and is co-localized with CASK in PSDs and in lipid rafts in brain. Further, parkin also associates with a large multimeric protein complex implicated in NMDA receptor signaling at the PSD. Importantly, these data suggest novel functions for parkin in synaptic transmission as well as neurodegeneration.

MATERIALS AND METHODS

Antibodies and DNA Constructs—The entire parkin coding region was PCR-amplified from a PC12 cDNA library (gift of Dr. Steven Morris, MNI) and subcloned into the *Eco*RI and *Not*I sites of pcDNA 3.1 (Invitrogen). The glutathione-S-transferase (GST)·Ubl-(1–103), GST·CT-(441–465), and GST·V/E-(441–465) fusion proteins were prepared by PCR amplifying and subcloning the corresponding fragments into pGEX-5X-1 (Amersham Biosciences). GST·NR2B was a gift from Dr. David Bredt (UCSF). The parkin antibody ASP5p was described previously (15). The anti-Ubl parkin antibody was raised in rabbit against the GST·Ubl fusion protein. The monoclonal synapsin antibody was a gift from Dr. Peter McPherson (MNI). The monoclonal Thy1.1 antibody was a gift from Dr. Erik Schweitzer (UCLA). Polyclonal antibodies to PICKI, GRIP1, and GRIP2 were a gift from Dr. Richard Huganir (Johns Hopkins). The polyclonal parkin antibody for immunocytochemistry was from Chemicon. Monoclonal synaptophysin, MAP2, and FLAG antibodies were from Sigma. The monoclonal anti-His antibody was from Novagen. Monoclonal PSD-95, CASK, flotillin, CAMKIIα, MUPP1, Homer1a, neurexin I, NR2B, GRIP, neurabin, and α-synuclein antibodies were from Transduction Laboratories.

Subcellular Fractionation of Rat Brain—Synaptosomes from whole rat brain were prepared by differential centrifugation as described (16). This yielded non-synaptic (P3, S3) and synaptosomal (P2) fractions. P2 was further fractionated into plasma membrane (LP1)-, synaptic vesicle (LP2)-, and cytosol (LS2)-enriched fractions. Synaptic plasma membrane (SPM) was prepared from LP1 using a discontinuous sucrose density gradient as described (17). Briefly, LP1 was brought to 1.1 M sucrose and placed under a 0.8 M/0.3 M sucrose step-gradient and centrifuged at 65,000 $\times$ *g* for 2.5 h. The SPM was recovered from the 0.8/1.1 M interface. Synaptic vesicles (SVs) were purified from LP2 by centrifugation at 120,000 $\times$ *g* for 4 h through a 50–800 mM linear sucrose density gradient, as described (16). Proteins were separated by SDS-PAGE, blotted onto nitocellulose, incubated with the indicated antibodies and imaged with enhanced chemiluminescence (Pierce).

Preparation of Rafts and PSDs—Rafts were isolated as described (18), with the following modifications. P2 was first purified on a discontinuous sucrose gradient (0.8 to 1.1 M) and pelleted at 100,000 $\times$ *g* for 1 h, followed by incubation in ice-cold 1% Triton X-100 for 20 min. The samples were adjusted to 40% sucrose, overlaid with a 30/0% sucrose step-gradient, centrifuged at 158,000 $\times$ *g* for 16 h, and equal volume fractions were collected from the bottom of the gradient. PSDs were isolated as described (13). Briefly, the purified P2 pellet was extracted in ice-cold 0.5% Triton X-100/50 mM Tris-HCl (pH 7.9) and centrifuged at 32,000 $\times$ *g* to obtain PSD I. PSD I was extracted a second time with either 0.5% Triton X-100 (PSD II) or with 3% sarkosyl (PSD III), followed by centrifugation at 200,000 $\times$ *g* for 1 h.

Primary Cortical Neuron Culture and Immunocytochemistry—Cortical neurons were isolated from the telecephalon of E16 mice (Charles River, Canada), triturated, and plated on poly-D-lysine-coated coverslips in Neurobasal medium supplemented with 0.5$\times$ N2, 0.5$\times$ B27 (Invitrogen), 100 μg/ml penicillin/streptomycin, and 2 mM glutamine. After 6 days *in vitro*, the coverslips were fixed in methanol for 10 min at −20 °C, blocked with 10% bovine serum albumin/phosphate-buffered saline and incubated with primary antibodies in 3% bovine serum albumin/phosphate-buffered saline for 1 h followed by secondary fluorochrome-conjugated antibodies for 1 h. Antibody concentrations: synaptophysin, 1:1000, MAP2, 1:800, CASK, 1:100, parkin (Chemicon), 1:1200. CY2-conjugated goat anti-mouse, and CY3-conjugated donkey anti-rabbit (Jackson Immunoresearch), 1:800. Slides were imaged using a Zeiss Axioskop microscope (Oberkochen, Germany) with a 100 $\times$ objective, using Northern Eclipse software.

GST Fusion Protein Affinity Chromatography, Immunoprecipitation, and Ubiquitination Assay—GST fusion proteins were expressed in *Escherichia coli* and immobilized on glutathione-Sepharose beads. For pull-downs, synaptosomes (P2) were solubilized with 1% deoxycholate, 50 mM Tris-HCl (pH 7.4) at 37 °C and cleared by centrifugation at 100,000 $\times$ *g* for 30 min. The supernatants (12 mg of protein) were incubated with immobilized GST fusion proteins overnight at 4 °C. The beads were washed three times with phosphate-buffered saline, and bound proteins were eluted with sample buffer at 65 °C. For immunoprecipitation, synaptosomes were solubilized as above, the supernatant (1 mg of protein) was brought to 1% Triton X-100/50 mM Tris-HCl and precleared for 1 h with protein A-Sepharose. Antibody was added to the supernatant and incubated overnight at 4 °C. The samples were incubated with protein A-Sepharose for 2 h, washed three times with 0.1% Triton X-100, 50 mM Tris-HCl, and bound proteins were eluted with sample buffer at 65 °C. For ubiquitination assays, immunoprecipitated parkin was resuspended in 50 μl of buffer containing 50 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 4 mM ATP, 10 μg of His-Ub (Sigma), 100 ng of human E1, and 2.4 μg of UbcH7 as an E2 (both from Affiniti Research). Reactions were incubated for 90 min at room temperature, terminated by adding SDS sample buffer, resolved by SDS-PAGE, and immunoblotted with anti-His and anti-CASK antibodies.

RESULTS AND DISCUSSION

Localization of Parkin to Synaptic Plasma Membrane but Not Synaptic Vesicles in Rat Brain—We used differential centrifugation to determine the subcellular localization of parkin in rat brain. Immunoblotting of the fractions with the anti-parkin antibody ASP5p, reveals that parkin occurs in both synaptic (P2) and non-synaptic (P3) fractions (Fig. 1*A*). Interestingly, at the synapse, parkin is highly enriched in membrane (LP1 and LP2) compared with cytosolic (LS2) fractions. Indeed, parkin co-fractionates with the AMPA receptor subunit GluR2/3, a plasma membrane marker, and synaptophysin, a synaptic vesicle (SV) marker, but not with α-synuclein, which is predominantly a cytosolic presynaptic protein (Fig. 1*A*). Finding that parkin co-sediments with particulate LP1 and LP2 fractions suggested that it is present in a synaptic membrane compartment. These fractions are however of relatively low purity and provide only crude localization information. To identify more precisely the subcellular localization of parkin, we used sucrose density gradient fractionation to further purify SPM and SVs from LP1 and LP2 respectively. In these purified preparations, parkin co-fractionates with the SPM markers GluR2/3 and Thy-1 (Fig. 1*B*) but not with the SV markers, synaptophysin, or synapsin (Fig. 1*C*). Interestingly, a recent report describes co-fractionation of parkin with SV markers, using different fractionation conditions (19). We find that parkin does not associate with SVs, an observation that is unlikely to result from fractionation conditions that removed parkin from SVs. Indeed, even small amounts of salt in the fractionation buffer readily remove certain SV proteins such as synapsin from SVs (16), but we used low salt conditions that did not displace synapsin from SVs (Fig. 1*C*). Parkin was recently found to bind and ubiquitinate the SV-associated protein CDCRel-1, although direct localization of parkin to SVs was not shown (11). Our findings do not preclude an interaction with CDCRel-1. Indeed, only a fraction of CDCRel-1 is associated with SVs (20) and the interaction with parkin could occur at a distinct site. Importantly, we find that parkin occurs on the SPM. This prompted us to investigate both the nature of the membrane association and whether parkin was targeted to a specific membrane specialization.

Association of Parkin with Synaptic Membranes Is Sensitive to High Ionic Strength but Not to Extraction with the Non-ionic Detergent Triton X-100—The sequence of parkin does not predict any transmembrane domains or lipid binding motifs. To characterize the nature of parkin's association with synaptic

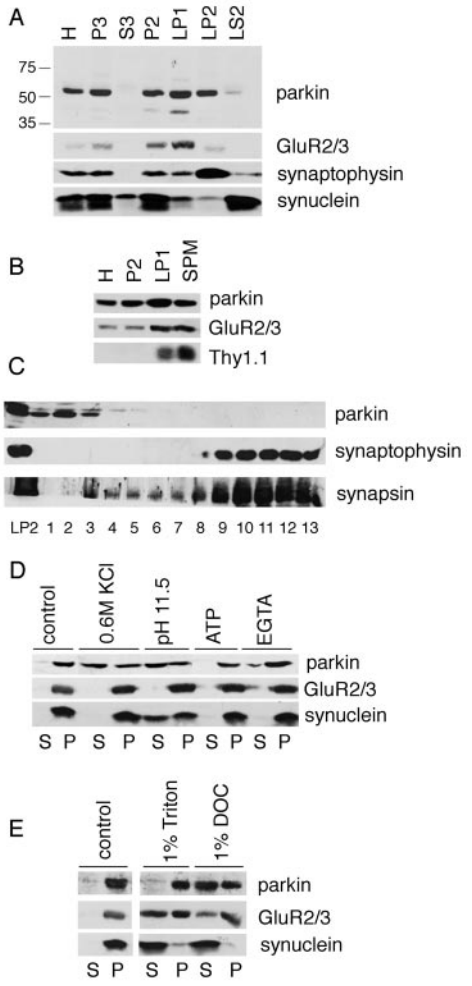

FIG. 1. **Parkin is localized to Triton X-100 insoluble microdomains at the synaptic plasma membrane.** *A*, Parkin is enriched in synaptic membrane fractions (LP1, LP2) in rat brain as shown by immunoblotting with the anti-parkin, ASP5p antibody. Antibodies against GluR2/3, synaptophysin and α-synuclein were used as plasma membrane, synaptic vesicle, and cytosolic markers, respectively. *H*, homogenate, *P3*, microsomal, *S3*, cytosolic, *P2*, synaptosomal, *LP1*, crude plasma membrane, *LP2*, crude synaptic vesicle, and *LS2*, synaptosomal cytosolic fractions. *B*, Parkin is localized to sucrose density gradient-purified SPM. GluR2/3 and Thy1.1 were used as plasma membrane markers. *C*, Parkin does not co-purify with sucrose density gradient-purified SVs. Parkin remained at the top of the gradient (*lanes 1–3*) whereas SVs localized to the middle fractions (*lane 8–13*) as shown by the SV markers synaptophysin and synapsin. *D*, Parkin's membrane association is electrostatic. LP1 was resuspended in either homogenization buffer alone (0.3 M sucrose/Hepes; control) or in buffer containing 0.6 M KCl, 0.1 M Na_2CO_3, pH 11.5, 2 mM ATP, or 2 mM EGTA. The samples were recentrifuged, and equal volumes of supernatant (*S*) and pellet (*P*) were analyzed with the ASP5p, GluR2/3, and α-synuclein antibodies. *E*, Synaptic plasma membrane parkin is insoluble in Triton X-100. LP1 was resuspended in homogenization buffer alone or in buffer containing either 1% Triton X-100 or 1% deoxycholate at 4 °C, recentrifuged and equal volumes of supernatant and pellet were analyzed as in *D*.

membranes, we resuspended LP1 pellets in either homogenization buffer alone or homogenization buffer containing high salt, Na_2CO_3 (pH 11.5), ATP, or EGTA. After re-pelleting the samples, we found that parkin was solubilized by raising ionic strength with high salt or by stripping the membrane with high pH. In contrast, none of the parkin was solubilized with ATP treatment and only a very small fraction was solubilized by Ca^{2+} chelation with EGTA (Fig. 1*D*). Our results suggest that the association of parkin with synaptic membranes is electrostatic rather than hydrophobic, possibly mediated by an interaction with another protein. In contrast, none of the treatments removed integral membrane proteins such as GluR2/3 from LP1 membranes, whereas high pH but not high salt removes α-synuclein from membranes (Fig. 1*D*). To further characterize the membrane association of parkin, we tested its ability to be solubilized by detergents. Pellets from LP1 were resuspended at 4 °C, in either homogenization buffer alone or homogenization buffer containing various detergents and re-pelleted. Interestingly, most of the parkin sedimented in the pellet after treatment with 1% Triton X-100 but not with 1% deoxycholate (Fig. 1*E*). In contrast, both α-synuclein and GluR2/3 were at least partially solubilized by both detergents. Importantly, resistance to solubilization in the non-ionic detergent Triton X-100 is a property of proteins within specialized microdomains of the plasma membrane, namely lipid rafts and PSDs, which serve as key sites for cell signaling and synaptic transmission (21, 22). Further, PDZ proteins have been found to be major components of these domains. Both the finding that SPM-associated parkin is resistant to extraction with Triton X-100 and the observation that a putative PDZ binding motif occurred at its C terminus prompted us to investigate further whether parkin interacts with PDZ proteins.

The C Terminus of Parkin Binds CASK—To identify PDZ proteins that bind parkin, we expressed a GST fusion protein encoding the C-terminal 24 amino acids of parkin (GST·CT) in *E. coli*. We also expressed GST alone, GST fused to the parkin Ubl domain (GST·Ubl), and GST fused to the C terminus of the NMDA receptor 2B subunit (GST·NR2B), which is known to interact with the first PDZ domain of PSD-95. As a further control for PDZ-mediated interactions, we expressed a GST fusion protein encoding the C terminus of parkin in which the last amino acid was changed from valine to glutamic acid (GST·V/E), a mutation that has been shown to disrupt PDZ-dependent interactions (23). The fusion proteins were used to pull-down interacting proteins from rat brain synaptosomes (P2). Bound proteins were eluted and analyzed by immunoblotting with a panel of antibodies raised against various PDZ proteins. GST·CT but not GST·Ubl or GST alone pulled-down the PDZ protein CASK, the mammalian homolog of *C. elegans* Lin-2 from brain extracts (Fig. 2*A*). In *C. elegans*, the three PDZ proteins, Lin-2, Lin-7, and Lin-10, are involved in targeting the receptor tyrosine kinase LET-23, an epidermal growth factor (EGF) receptor homolog, to the basolateral membrane in vulval epithelial cells (24). The C terminus of LET-23 binds the class I PDZ domain of Lin-7, which in turn forms a complex with Lin-2 and Lin-10. Importantly, CASK/mLin-2, Veli/mLin-7, and Mint/mLin-10, the mammalian homologs of Lin-2, Lin-7, and Lin-10, also form a tight tripartite complex that is expressed at high levels in brain (25). As in *C. elegans*, the interactions between CASK, Veli, and Mint do not involve the PDZ domains, leaving them free to interact with other proteins. The class II PDZ domain of CASK has been shown to interact with the C terminus of neurexin, the receptor for α-latrotoxin, the black widow spider venom (14) as well as syndecan, a cell surface heparan sulfate proteoglycan (13). Importantly, the sequence FDV, at the C terminus of parkin is highly reminiscent of motifs found to interact with CASK (26). Further, the interaction between CASK and parkin appears to be PDZ-dependent, because the valine to glutamic acid mutation in GST·V/E abolishes binding (Fig. 2*A*). None of the other PDZ proteins we tested, including several with type II PDZ domains such as PICK1, GRIP1 & 2, Tiam1, bound the C terminus of parkin, demonstrating the specificity of the parkin-CASK interaction (Fig. 2*B*). Similarly, PSD-95 was efficiently pulled-down with GST·NR2B but not with GST·CT. Together, these

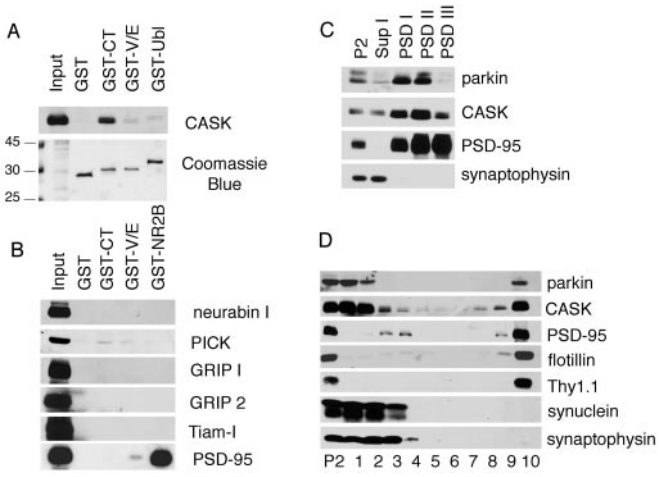

FIG. 2. **The C terminus of Parkin binds CASK and both Parkin and CASK are co-localized in PSDs and in lipid rafts.** *A* and *B*, solubilized synaptosomes were incubated with either GST, a GST fusion of the C terminus of parkin (GST·CT), a GST fusion with a terminal valine to glutamic acid mutation (GST·V/E), a GST fusion of N-terminal Ubl domain of parkin (GST·Ubl) or a GST fusion of the C terminus of the NMDA receptor subunit 2B (GST·NR2B). Coomassie Blue staining shows that equivalent amounts of GST fusion proteins were used. Bound proteins were analyzed by immunoblotting with antibodies against CASK (*A*) or the indicated PDZ protein (*B*). The *input* contains 10% of the extract used for binding. *C*, Parkin and CASK are co-localized at the PSD. *P2*, purified synaptosomes; *Sup I*, supernatant after the first 0.5% Triton X-100 extraction; *PSD I*, pellet after the first Triton extraction; PSD II, pellet after the second Triton extraction; PSD III, pellet after the Triton followed by 3% sarkosyl extractions. Parkin and CASK are co-localized in PSD II along with the PSD marker PSD-95. *D*, Parkin and CASK are co-localized in low density lipid rafts. Triton X-100-resistant lipid rafts were isolated from purified synaptosomes using a discontinuous flotation gradient. Parkin is found in the low density raft fraction (*lane 10*) along with CASK and the raft markers flotillin and Thy1.1.

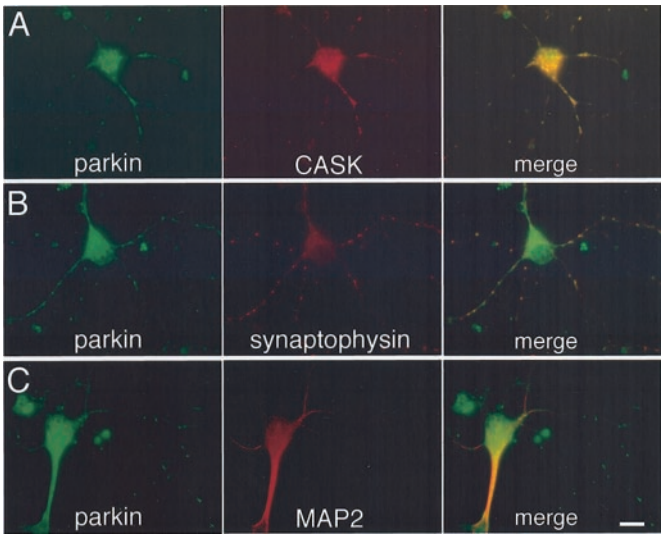

FIG. 3. **Parkin co-localizes with CASK in cortical neurons.** Cortical neurons were double immunolabeled with antibodies against parkin (*left*) and CASK (*middle*) (*A*); Parkin (*left*) and the synaptic marker synaptophysin (*middle*) (*B*); parkin (*left*) and the somato-dendritic marker MAP2 (*middle*) (*C*). The merged images (*right*) demonstrate the extensive co-localization of parkin with CASK and the partial co-localization with synaptophysin in processes (*right*). In contrast, the punctate parkin staining in cell bodies and processes does not co-localize with MAP2. *Scale bar*, 10 μm.

data indicate that the C terminus of parkin binds CASK specifically via a type II PDZ-mediated interaction.

Parkin Co-Localizes with CASK in Postsynaptic Densities and Lipid Rafts in Brain—CASK has been shown previously to occur in both synaptic and non-synaptic membranes and to be enriched in PSDs (13). The finding that the C terminus of parkin binds CASK prompted us to ask whether parkin was co-localized with CASK in Triton X-100-insoluble, plasma membrane microdomains, such as PSDs and lipid rafts. We prepared PSD fractions from rat brain by sequential Triton X-100 and Sarkosyl extractions of purified synaptosomes. After the first Triton X-100 extraction, both parkin and CASK were enriched in the pellet (PSD I) compared with the supernatant (Sup I), as was PSD-95, the prototypic PSD marker (Fig. 2*C*). In contrast, synaptophysin was efficiently extracted with Triton X-100 and found predominantly in the supernatant. Importantly, essentially all the parkin and CASK were recovered in the pellet after the second extraction with Triton X-100 (PSD II), suggesting they occur in the PSD along with PSD-95. However, in contrast to PSD-95, which is associated with the PSD core (PSD III), most of the parkin and CASK were solubilized by sarkosyl, suggesting a more peripheral association with the PSD, as shown previously for CASK and other PSD proteins (13, 23). Further, because neither parkin nor CASK is exclusively synaptic, we were also interested in whether they occurred together in other Triton X-100-insoluble membrane compartments, such as lipid rafts.

Lipid rafts were prepared by extracting purified synaptosomes with ice-cold Triton X-100 followed by flotation through a sucrose gradient. Importantly, we find that both parkin and CASK floated to the same low density fraction as the prototypic brain raft markers flotillin and Thy-1 (27, 28) (Fig. 2*D*). Further, PSD-95 also floated to the same fraction as parkin, CASK, Thy-1, and flotillin, consistent with its previous localization in raft-like membranes in brain (29). In contrast, the two synaptic proteins, synaptophysin and α-synuclein remained at the bottom of the gradient, validating the purity of our raft preparation. Rafts represent specialized subdomains of the plasma membrane, which appear to function as platforms for integrating cellular information at the cell surface (21). Further, rafts are involved in sorting a subset of proteins such as Thy-1 to axonal membranes in polarized hippocampal neurons (28). Whereas our identification of CASK in rafts is novel, other synaptic PDZ proteins such as PSD-95 and GRIP, have been found in lipid rafts previously, suggesting a functional link between the PSD and lipid raft compartments (18, 29). Our finding of parkin and CASK in lipid rafts may have special relevance for neurodegenerative disease. Indeed, amyloid precursor protein (APP), presenilin, and prion protein (PrP) have all been localized to lipid rafts (30, 31). Further, both the processing of APP into the amyloidogenic Aβ42 fragment and the conversion of normal PrP^c into the scrapies isoform PrP^{sc}, appear to occur within rafts. Whether parkin or CASK is implicated in these processes remains to be determined. It is interesting to note, however, that the CASK partner, Mint has been implicated in APP processing (32). Importantly, our finding that parkin and CASK are co-localized in PSDs and lipid rafts further substantiates the biological relevance of their PDZ-mediated interaction and prompted us to examine whether they are also co-localized in cultured cortical neurons.

Parkin Is Co-localized with CASK in Cortical Neurons—To determine the subcellular localization of parkin in cortical neurons, we prepared primary cultures from E16 rat cortex and used immunofluorescence with double labeling for parkin and various markers. Consistent with our subcellular fractionation data above (Fig. 1*A*), endogenous parkin staining appeared punctate in neuronal processes and cell bodies, suggesting parkin associates with membranes (Fig. 3). This contrasted markedly with the more diffuse staining pattern seen with the somato-dendritic marker, MAP2 (Fig. 3*C*). Further, a signifi-

cant portion of the punctate parkin staining in processes co-localized with synaptophysin, again supporting a localization of parkin at synaptic terminals (Fig. 3*B*). Interestingly however, whereas a portion of the parkin staining, especially in cell bodies, did not co-localize with synaptophysin, CASK staining extensively overlapped with parkin, in both neuronal processes and in cell bodies (Fig. 3*A*). Importantly, the finding that endogenous parkin is exquisitely co-localized with endogenous CASK in cortical neurons further supports the biologic relevance of the PDZ-mediated parkin-CASK interaction. The finding that the C terminus of parkin binds CASK and that they have a very similar subcellular distribution prompted us to examine whether the proteins also interact *in vivo* in brain and whether parkin ubiquitinates CASK.

CASK Associates with Parkin in Rat Brain but Is Not a Substrate for Parkin-mediated Ubiquitination—We were unable to immunoprecipitate parkin with the ASP5p antibodies used above. We therefore raised a new antibody against the Ubl domain of rat parkin (Ubl^{1-103}), which recognizes a ~52-kDa band, the predicted size of parkin, in both whole brain lysate and parkin-transfected COS-7 cells (Fig. 4*A*). This band is eliminated when the antibody is pre-absorbed with the immunogen and importantly, the Ubl^{1-103} antibody immunoprecipitated parkin (Fig. 4*B*). To identify PDZ proteins associated with parkin *in vivo*, we used the Ubl^{1-103} antibody to immunoprecipitate parkin from rat brain synaptosomes (P2). The Ubl^{1-103} antibody efficiently co-immunoprecipitated CASK suggesting that parkin interacts with CASK *in vivo* (Fig. 4*C*). Parkin has been shown to function as an E3 Ub-ligase, but to date only three substrates of parkin-mediated ubiquitination have been identified (6, 11, 33). To determine whether parkin also ubiquitinates CASK, we carried out an *in vitro* ubiquitination assay by incubating parkin, immunoprecipitated from brain lysates, with His-tagged Ub, recombinant E1, and UbcH7, an E2 known to support parkin's E3 ubiquitin ligase activity (10), in the presence of ATP. High molecular weight, His-ubiquitinated proteins were observed in the presence of parkin, His-Ub, E1, UbcH7, and ATP but not in control reactions lacking either parkin or UbcH7, confirming that parkin exhibited E2-dependent Ub-ligase activity in our assay (Fig. 4*D*). Interestingly, despite the robust co-immunoprecipitation of CASK with parkin, the electrophoretic mobility of CASK was not altered, suggesting it is not ubiquitinated by parkin (Fig. 4*D*). The precise role of CASK in parkin-mediated ubiquitination therefore remains to be determined, but may involve trafficking the E3 Ub-ligase to the appropriate subcellular compartments, a role consistent with CASK's previously described function (24, 34).

Parkin Is Part of a Large Multimeric Protein Complex Implicated in Synaptic Transmission—The finding that parkin occurred at the PSD suggested it may also interact with proteins other than CASK at this site. Interestingly, despite the absence of direct binding to the C terminus of parkin (Fig. 2*B*), parkin robustly co-immunoprecipitated the PDZ protein PSD-95 as well as the NMDAR NR2B subunit, Calcium and calmodulin-dependent protein kinase II (CaMKII) and homer 1a (Fig. 4*C*). These proteins are all known to associate as part of a large multimeric complex, implicated in NMDA trafficking, scaffolding, and signaling at the PSD (35). Our findings suggest that parkin interacts with this protein complex, possibly via CASK. In contrast, neither MUPP1, which contains 13 PDZ domains, nor the PDZ protein neurabin I were co-immunoprecipitated with parkin. Similarly, neither neurexin I, which binds the PDZ domain of CASK (14), nor α-synuclein were co-immunoprecipitated with parkin (Fig. 4*C*). We do not know, however, whether an *O*-glycosylated form of α-synuclein, which has recently been shown to be a parkin substrate (6), is co-immunoprecipitated with our Ubl^{1-103} antibody. Importantly, these findings suggest that parkin interacts specifically with CASK and the NMDAR complex but not with other PDZ and non-PDZ proteins in brain. Although it is not known whether CASK and the NMDAR complex, in turn, interact with each other, they both occur at the PSD and the PDZ domain of Veli binds the C terminus of the NMDA receptor NR2 subunit, possibly providing a link between the two complexes (36). Further, a PDZ domain of Mint binds the C terminus of KIF17, a kinesin superfamily molecular motor, suggesting a role for the CASK/Mint/Veli complex in dendritic transport of NMDA receptor-containing vesicles along micotubules (34). Although CASK itself does not appear to be ubiquitinated by parkin (Fig. 4*D*), it may also function in trafficking parkin or in providing a scaffold for parkin-mediated ubiquitination of other proteins within the NMDA receptor complex. Importantly, we have

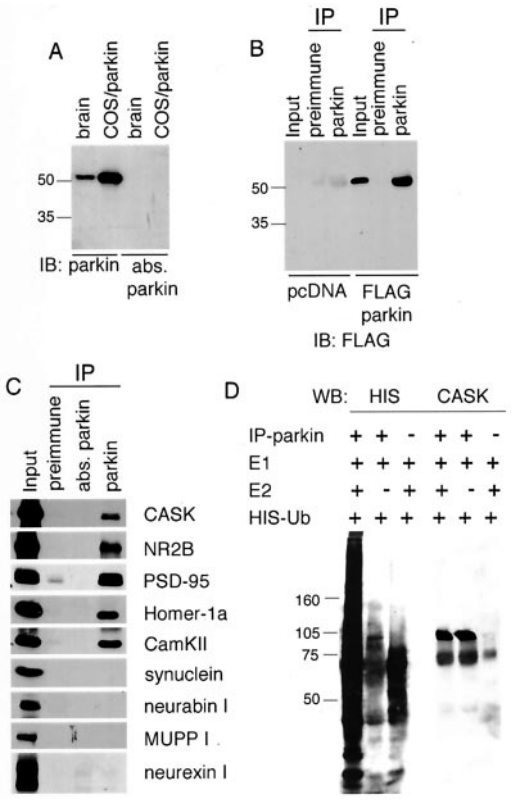

FIG. 4. **Parkin co-immunoprecipitates CASK and components of the NMDA receptor-signaling complex from brain.** *A* and *B*, characterization of anti-parkin Ubl^{1-103} antibody. *A*, anti-Ubl^{1-103} recognizes a single band at 52 kDa in both brain lysates and in parkin-transfected COS-7 cells. Pre-absorption of anti-Ubl^{1-103} with GST·Ubl eliminates this band. *B*, COS-7 cells were transfected with pcDNA 3.1 or FLAG-parkin, and lysates were immunoprecipitated with either preimmune serum or with anti-Ubl^{1-103}. FLAG-parkin was specifically immunoprecipitated from COS-7 cells with anti-Ubl^{1-103} as shown by immunoblotting with anti-FLAG. Immunoprecipitation of parkin from brain is not shown as it co-migrated with the Ig heavy chain. *C*, immunoprecipitation of detergent-solubilized synaptosomes using anti-Ubl^{1-103}. The immunoprecipitates were immunoblotted and analyzed with antibodies against CASK and other synaptic proteins as indicated. Preimmune serum and Ubl^{1-103} absorbed with GST·Ubl were used as controls. The *input* contains 10% of the brain lysate used for immunoprecipitation. *D*, endogenous parkin from rat brain exhibits E2-dependent ubiquitin-ligase activity *in vitro* but does not ubiquitinate CASK. Parkin was immunoprecipitated from rat brain using anti-Ubl or pre-absorbed anti-Ubl and incubated with E1, His-Ub, ATP, with or without the E2 UbCH7. His-ubiquitinated proteins were observed by immunoblotting with anti-His. Immunoblotting with anti-CASK reveals that CASK is present in the parkin immunoprecipitated complex but is not modified by Ub. The *asterisk* denotes a nonspecific band.

shown, for the first time, that a PDZ-mediated interaction is implicated in the pathogenesis of PD. We have also shown that parkin, an E3 Ub-ligase, is localized to lipid rafts and the PSD suggesting a novel role for ubiquitination in these subcellular compartments. Indeed, the potential for parkin-mediated ubiquitination to regulate key synaptic proteins, such as those found in the CASK and NMDAR complexes, has important implications for our understanding of both synaptic transmission and plasticity.

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