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ARTICLE *in* JOURNAL OF NEUROSCIENCE RESEARCH · MAY 2012

Impact Factor: 2.59 · DOI: 10.1002/jnr.22828 · Source: PubMed

Journal of Neuroscience Research

Journal of Neuroscience Research 90:1030–1042 (2012)

Neddylation Positively Regulates the Ubiquitin E3 Ligase Activity of Parkin

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Mutations in the parkin gene underlie a familial form of Parkinson's disease known as autosomal recessive juvenile Parkinsonism (AR-JP). Dysfunction of parkin, a ubiquitin E3 ligase, has been implicated in the accumulation of ubiquitin proteasome system-destined substrates and eventually leads to cell death. However, regulation of parkin enzymatic activity is incompletely understood. Here we investigated whether the ubiquitin E3 ligase activity of parkin could be regulated by neddylation. We found that parkin could be a target of covalent modification with NEDD8, a ubiquitin-like post-translational modifier. In addition, NEDD8 attachment caused an increase of parkin activity through the increased binding affinity for ubiquitin-conjugating E2 enzyme as well as the enhanced formation of the complex containing parkin and substrates. These findings point to the functional importance of NEDD8 and suggest that neddylation is one to the diverse modes of parkin regulation, potentially linking it to the pathogenesis of AR-JP.

Key words: parkin; ubiquitin E3 ligase; NEDD8; neddylation; Parkinson's disease

Parkinson's disease (PD) is a major neurodegenerative disease characterized by a distinct set of movement disorders, such as muscle rigidity, tremor, and bradykinesia (Lang and Lozano, 1998). The pathologic hallmarks of PD include the progressive degeneration and death of dopaminergic neurons in the substantia nigra pars compacta as well as the presence of cytoplasmic inclusions known as Lewy bodies (LBs). The etiology of PD remains poorly understood, but several genetic loci have been implicated in the pathogenesis of familial forms of PD. Discovery of these loci has considerably advanced our understanding of the pathogenesis of familial PD.

Included among these loci is *parkin*, mutation of which is responsible for early-onset autosomal recessive juvenile Parkinsonism (AR-JP). AR-JP is characterized by early disease manifestation, relatively slow disease progression, and a good long-term response to levodopa therapy. Parkin functions in the ubiquitin-proteasome system as an E3 ubiquitin-protein ligase, together with the E2 ubiquitin-conjugating enzymes UbcH7 or UbcH8 (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). Given the functional role of parkin, one might surmise that the loss of parkin function results in the gradual and abnormal accumulation of its substrates, which may then induce neuronal cell death and the familial PD syndrome. Several substrates of parkin have been reported, including synphilin-1, Pael-R, α-synuclein, p38 subunit of aminoacyl-tRNA synthetase, and RanBP2 (Chung et al., 2001; Imai et al., 2001; Shimura et al., 2001; Corti et al., 2003; Ren et al., 2003; Staropoli et al., 2003; Tsai et al., 2003; Um et al., 2006).

Neural precursor cell-expressed developmentally downregulated gene 8 (NEDD8) is a small, ubiquitin-like protein that is 60% identical and 80% homologous to ubiquitin (Kamitani et al., 1997). NEDD8 becomes covalently linked to its substrates in a manner analogous to ubiquitination. This modification, known as *neddylation*, occurs when NEDD8 is activated by APPBP1-Uba3, a heterodimeric E1 enzyme, and is then conjugated to lysine by the E2 enzyme Ubc12 (Gong and

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: Brain Research Center of the 21st Century Frontier Research Program Technology funded by the Ministry of Education, Science and Technology (MEST) of the Republic of Korea; Contract grant number: 2009K-001251 (to K.C.C.); Contract grant sponsor: National Research Foundation (NRF) of Korea funded by MEST; Contract grant number: 2010-0018916 (to K.C.C.); Contract grant sponsor: Basic Science Research Program through NRF; Contract grant number: 2011-0001176 (to K.C.C.); Contract grant sponsor: Korea Health 21 R&D Project, Ministry of Health, Welfare and Family Affairs; Contract grant number: A092004 (to K.C.C.).

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Received 24 August 2011; Revised 8 October 2011; Accepted 12 October 2011

Published online 24 January 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.22828

Yeh, 1999). Ultimately, an exposed C-terminal glycine present on the mature form of NEDD8 forms an isopeptide bond with a lysyl residue of receptive substrates. The best known substrates of NEDD8 conjugation are the cullin family members, particularly cullin1. Neddylation of this factor, which is a component of the SCF (Skp-1, Cullin1, F-box) ubiquitin ligase complex, plays an important role in the ubiquitin ligase function of some SCF complexes (Read et al., 2000; Wu et al., 2006; Wu et al., 2000). A recent report revealed that p53 (MDM2 oncogene product), p73 (p53 homologue), von Hippel-Lindau protein, epidermal growth factor receptor, breast-cancer-associated protein 3, and ribosomal proteins are potentially neddylated (Stickle et al., 2004; Xirodimas et al., 2004, 2008; Gao et al., 2006; Oved et al., 2006; Watson et al., 2006). Like other types of covalent modification, neddylation alters the cellular function and/or localization of target proteins (Rabut and Peter, 2008; Xirodimas, 2008).

NEDD8 has been observed in ubiquitinated inclusion bodies, including LBs in PD (Corti et al., 2003). Moreover, recent data showed that NEDD8 is present in ubiquitin-positive neuronal and glial inclusions of several neurodegenerative diseases (Mori et al., 2005). These data suggest that NEDD8 and defects in neddylation may be linked to the pathogenesis of some neurodegenerative disorders, including PD. This, taken with the fact that parkin dysfunction underlies a forms of familial PD, prompted us to investigate the potential regulation of parkin by NEDD8. The present study demonstrated that parkin is a target for in vivo covalent modification of NEDD8. In addition, neddylation stimulated the ubiquitin E3 ligase activity of parkin, effects attributable to the enhanced recruitment of ubiquitin-conjugating E2 enzyme and substrates to parkin.

MATERIALS AND METHODS

Materials

Peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Zymed Laboratories (South San Francisco, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and LipofectAmine Plus reagents were obtained from Invitrogen (Carlsbad, CA). MG132 was purchased from A.G. Scientific (San Diego, CA). Anti-HA, anti-GFP, anti-GST, anti-Hsp90, anti-ubiquitin, anti-α-tubulin, antihistone H1, and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Flag antibody, G-418, and cycloheximide were from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-T7 antibody was purchased from Covance (Princeton, NJ), and mouse monoclonal anti-T7 antibody was from Novagen (Madison, WI). Monoclonal anti-parkin, polyclonal anti-NEDD8, and polyclonal anti-Ubc12 antibodies were purchased from Cell Signaling (Beverly, MA).

The mammalian expression vectors encoding for Myc-tagged human parkin (pcDNA3.1-Myc-parkin) and Flag-tagged parkin were kindly provided by K. Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and G. Lee (Ajou University, Suwon, Korea), respectively. Plasmids encoding bacterial recombinant GST fused with wild-type and deleted parkin (77–465, 1–170) were gifts from H. Rhim (The Catholic University, Seoul, Korea), and those encoding T7-tagged NEDD8 and NEDD8-ΔGG were provided by M. Ohh (University of Toronto, Toronto, Ontario, Canada). Plasmids encoding GFP-tagged NEDD8 and NEDD8-ΔGG were kindly provided by C.Y. Choi (Sungkyunkwan University, Suwon, Korea), and plasmid encoding Myc-tagged p38 was kindly provided by S. Kim (Seoul National University, Seoul, Korea).

Cell Culture and Preparation of Cell Lysates

Human embryonic kidney 293 (HEK293), monkey kidney fibroblast (Cos7), murine neuroblastoma N2a, rat embryonic hippocampal progenitor (H19-7), and dopaminergic neuroblastoma SH-SY5Y cells were maintained in DMEM containing 10% FBS and 100 U/ml penicillin-streptomycin. For each preparation of cell lysates, cells were rinsed twice with ice-cold PBS and solubilized in lysis buffer containing 50 mM Tris (pH 7.4), 1.0% Nonidet P-40, 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Next, 1% NP-40-soluble and -insoluble fractions were obtained by lysing cells in lysis buffer containing 1% NP-40. The resulting lysates were sedimented at 16,000*g* for 30 min at 4°C, and the supernatants were collected (S fraction). The remaining pellets were washed once with PBS, re-extracted with SDS buffer containing 2% SDS, and sedimented at 16,000*g* for 30 min at 4°C. The resulting supernatant (P fraction) was then collected. For neddylation assay, cells were rinsed twice with ice-cold PBS and solubilized in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% SDS, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, and 0.2 mM PMSF.

Generation of SH-SY5Y Cell Lines Stably Expressing Parkin

Generation of SH-SY5Y cells stably overexpressing parkin has been previously described (Um et al., 2009). Briefly, SH-SY5Y cells were transfected with pcDNA3.1 plasmid expressing Myc-tagged wild-type parkin using LipofectAmine Plus reagents. Two days later, stably transfected cells were selected in DMEM containing 2 mg/ml of G-418. All positive cell lines used for the experiments described here were maintained in DMEM containing 500 g/ml of G-418 to prevent extrusion of the integrated constructs.

Immunocytochemistry

Cells were fixed in 3.7% formaldehyde in PBS solution, washed twice with PBS, permeabilized in 0.2% Triton X-100 in PBS, blocked with 1% BSA, and incubated with an appropriate dilution of primary antibody. After being washed twice with PBS, cells were incubated with secondary antibodies conjugated to TRITC or FITC. The cells were then subjected several additional PBS washes, and mounted in Slow Fade Light Antifade reagent with DAPI (Invitrogen, Eugene,

OR). Immunostained cells were observed using a Carl Zeiss LSM-510 Meta confocal microscope.

Immunoprecipitation

One microgram of suitable antibodies was incubated overnight at 4°C with 1 mg of cell extracts prepared in lysis buffer. The mixtures was then incubated with 30 μl of a 1:1 suspension of protein A-Sepharose beads for 2 hr at 4°C with gentle rotation. The beads were pelleted and washed five times with cell lysis buffer. The immunocomplexes were dissociated by being boiled in SDS-PAGE sample buffer. Protein samples were then separated by SDS-PAGE and transferred onto nitrocellulose membranes, which were subsequently blocked for 1 hr at room temperature in TBST buffer containing 20 mM Tris (pH 7.6), 137 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk. The membranes were then incubated overnight at 4°C in 3% nonfat dry milk containing the appropriate primary antibodies, washed several times in TBST, and incubated with horseradish peroxidase-coupled secondary antibody for 60 min. The membrane was washed several times with TBST, and secondary signals were visualized with ECL reagents (Perkin Elmer).

Preparation of Cytosolic and Nuclear Fractions

Cells were washed with ice-cold PBS and incubated for 30 min on ice in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) supplemented with protease inhibitors, including dithiothreitol (DTT), aprotinin, and leupeptin. The cells were then lysed with a disposable syringe and centrifuged at 1,000*g* for 15 min at 4°C. The resulting supernatants (cytosolic fractions) were collected and clarified by centrifugation 15,000*g* for 15 min at 4°C. The remaining nuclear pellets were washed with hypotonic buffer, lysed in 1.0% NP-40 lysis buffer, and clarified (15,000 g for 15 min at 4°C) to isolate nuclear fraction (supernatant).

GST Pull-Down Assay

GST pull-down assays were performed by incubating GST or GST-fused Ubc12 immobilized on GST beads for 2 hr or overnight at 4°C with lysates from cells overexpressing either Myc-tagged parkin or Myc-MDM2, respectively. The mixtures were then successively washed with wash buffer I (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 30 mM $MgCl_2$, 40 mM NaCl, 1% Nonidet P-40) and wash buffer II (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 30 mM $MgCl_2$, 40 mM NaCl). Finally, the bound proteins were eluted with 2× SDS buffer, separated by SDS/PAGE, and subjected to Western blot analysis using anti-Myc antibody.

Assessment of Cell Viability

Cell viability was quantified using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, cells stably expressing parkin or parental cells were seeded in 24-well plates, transfected with T7-NEDD8 or T7-NEDD8-ΔGG for 24 hr, and either left untreated or stimulated with 200 μM 1-methyl-4-phenylpyridinium ion (MPP^+) for an additional 24 hr. The medium was then removed, and 20 μl of CCK-8 solution was added to each well. The plate was incubated for 2 hr at 37°C, and the absorbance was measured at 450 nm using a microplate reader.

RESULTS

Overexpressed Parkin Is Neddylated in Mammalian Cells

To determine whether parkin can be modified by NEDD8 in mammalian cells, we transfected HEK293 cells with plasmid encoding Myc-tagged parkin alone or together with either T7-tagged NEDD8 or a T7-tagged mutant form of NEDD8 missing the essential C-terminal glycine-glycine residues required for conjugation (NEDD8-ΔGG). The cells were lysed with 1% SDS buffer to eliminate the possibility that parkin binds to intracellular neddylated proteins. As shown in Figure 1A, at least four different slowly migrating forms of parkin were observed in cells cotransfected with Myc-parkin and wild-type T7-NEDD8. However, no bands were detected in cells transfected with NEDD8 alone, Myc-parkin alone, or both Myc-parkin and NEDD8-ΔGG (Fig. 1A). Multiple slowly migrating parkin bands were also observed upon cotransfection of Myc-parkin and GFP-fused NEDD8 (Fig. 1B). As expected, cotransfection of GFP-fused conjugation-defective NEDD8 with Myc-parkin failed to produce any bands. These results indicate that parkin is covalently modified by NEDD8 in mammalian cells. In accordance with this finding, confocal analysis of HEK293 cells (Fig. 1C) and neuroblastoma N2a cells (Fig. 1D) that were stained with T7 or Myc antibodies revealed that T7-NEDD8 and Myc-parkin were colocalized, with these proteins predominantly cytoplasmic.

Endogenous Parkin Is Neddylated in Neuronal Cells

To extend our findings with overexpressed proteins, we investigated whether endogenous parkin is modified with endogenous NEDD8 in neuroblastoma SH-SY5Y cells. Coimmunoprecipitation (co-IP) assay revealed that endogenous parkin was modified by NEDD8 (Fig. 2A,B). To verify the identity of this band further, the knockdown effects of parkin, NEDD8, and Ubc12 were assessed. Immunoblot analysis of SH-SY5Y cells proved that each siRNA specifically and individually reduces the expression of target protein (Supp. Info. Fig. 1). When cells were transfected with siRNA against NEDD8 (Fig. 2C) or parkin (Fig. 2D), the level of neddylated endogenous parkin band was significantly reduced, but this was not seen with nonspecific siRNA as a control. Interestingly, the amount of modified parkin bands was not considerably affected by the knockdown of Ubc12 (data not shown), which could be explained as there possibly being other compensatory E2 enzymes for parkin neddylation or as the Ubc12 not being completely abolished, and the small amount of residual Ubc12 enzyme efficiently induced the parkin neddylation. In addition, confocal microscopic analysis

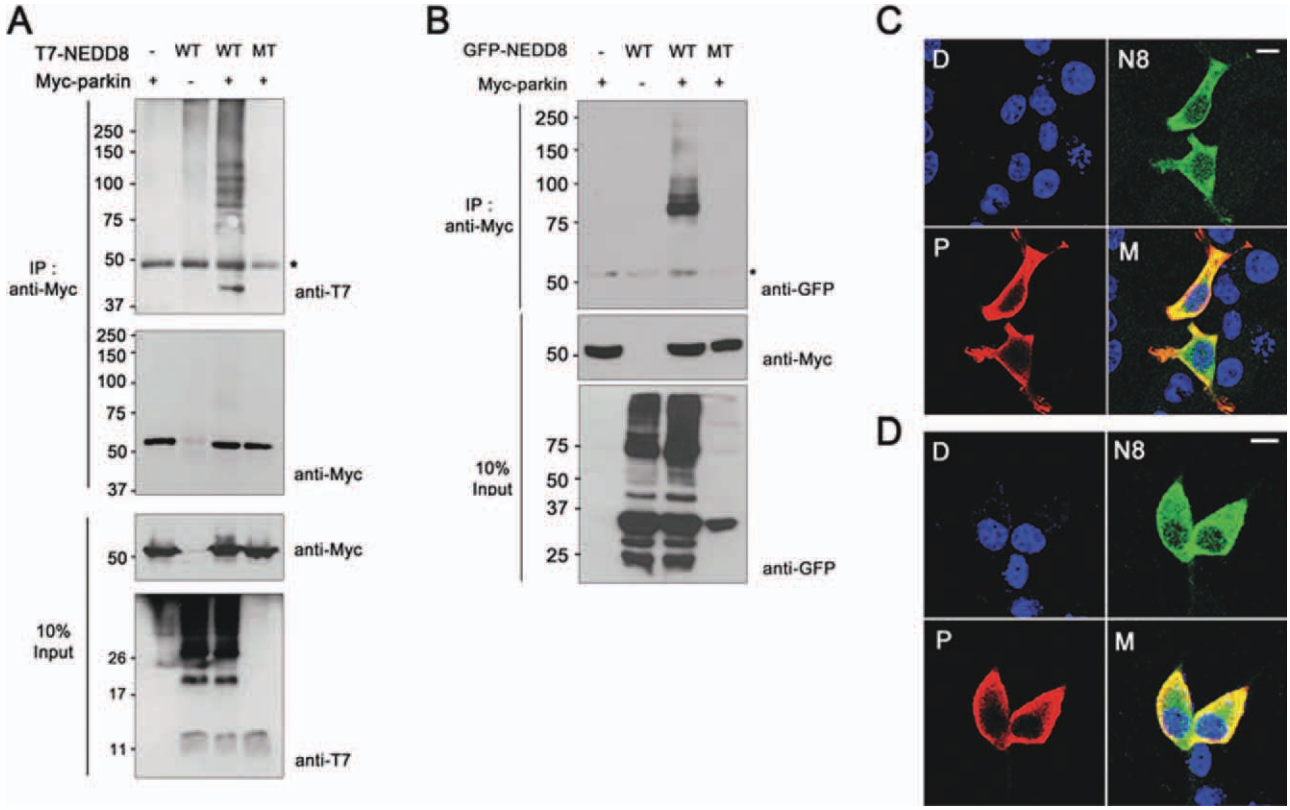

Fig. 1. Overexpressed parkin is neddylated in mammalian cells. **A,B:** HEK293 cells were transfected with Myc-tagged parkin ± T7-tagged (A) or GFP-tagged (B) forms of NEDD8 (WT) or NEDD8-ΔGG (MT). Cells were lysed in buffer containing 1% SDS. Anti-Myc coimmunoprecipitates were then subjected to Western blot analysis using anti-T7 (A) or anti-GFP (B) antibodies. Proper expression of the target proteins was determined by probing whole-cell lysates with anti-Myc (A,B), anti-T7 (A), and anti-GFP (B) antibodies. Asterisks indicate IgG heavy chains. **C,D:** Confocal images showing immunocytochemical staining for T7-tagged NEDD8 (N8) and Myc-tagged parkin (P) in HEK293 (C) and N2a (D) cells. Cells were labeled and counterstained with DAPI at 24 hr after transfection. These data are representative of three independent experiments. Scale bars = 10 μm.

demonstrated that parkin was colocalized with NEDD8 in the cytoplasm of the SH-SY5Y neuroblastoma cell line (Fig. 2E). Neddylated parkin was localized predominantly in the cytoplasm, whereas only very small amounts were detected in the nucleus (Fig. 2E). The specificity of the antibodies used for Figure 2E was also verified by using siRNA against either parkin or NEDD8 and nonspecific control siRNA (Supp. Info. Fig. 2). As shown in Supporting Information Figure 1, pretreatment with siRNAs against parkin or NEDD8 specifically decreased the level of parkin or NEDD8, respectively, but not with nonspecific control siRNA. These data argue against the idea that covalent attachment of NEDD8 to parkin is an artifact arising from ectopic DNA transfection and suggest that endogenous parkin is neddylated in neuronal cells.

Multiple Sites in the Parkin Molecule Are Likely Targeted for Covalent NEDD8 Attachment

To identify the exact NEDD8 modification site(s) in parkin, we analyzed neddylation of several parkin deletion mutants using in vivo neddylation assays. We found that parkin$^{1–415}$, parkin$^{77–465}$, and wild-type parkin were modified with NEDD8, whereas parkin$^{1–100}$ and parkin$^{295–465}$ were not (Supp. Info. Fig. 3A,B). These results suggest that NEDD8 binds to the region of parkin spanning amino acids 101–294. This putative targeting region contains five lysine residues that may be neddylated (Supp. Info. Fig. 3C). To identify the exact targeting site(s), we separately mutated two lysine residues (K151 and K220) to arginine. Neddylation assay using these two mutants revealed that they retained the ability to be neddylated (Supp. Info. Fig. 3D). Interestingly, mutation of the other three lysine residues to arginine (K129R, K161R, and K211R) produced mutants that were neddylated to the same extent as wild-type parkin (Supp. Info. Fig. 3D,G). Neddylation also was not significantly altered by mutation of more than two sites at once (Supp. Info. Fig. 3D). This was also true of arginine substitution of all five residues at once (K129R/K151R/K161R/K211R/K220R; Supp. Info. Fig. 3D,G). These results indicate that NEDD8-targeted residues are not simply limited to the region spanning

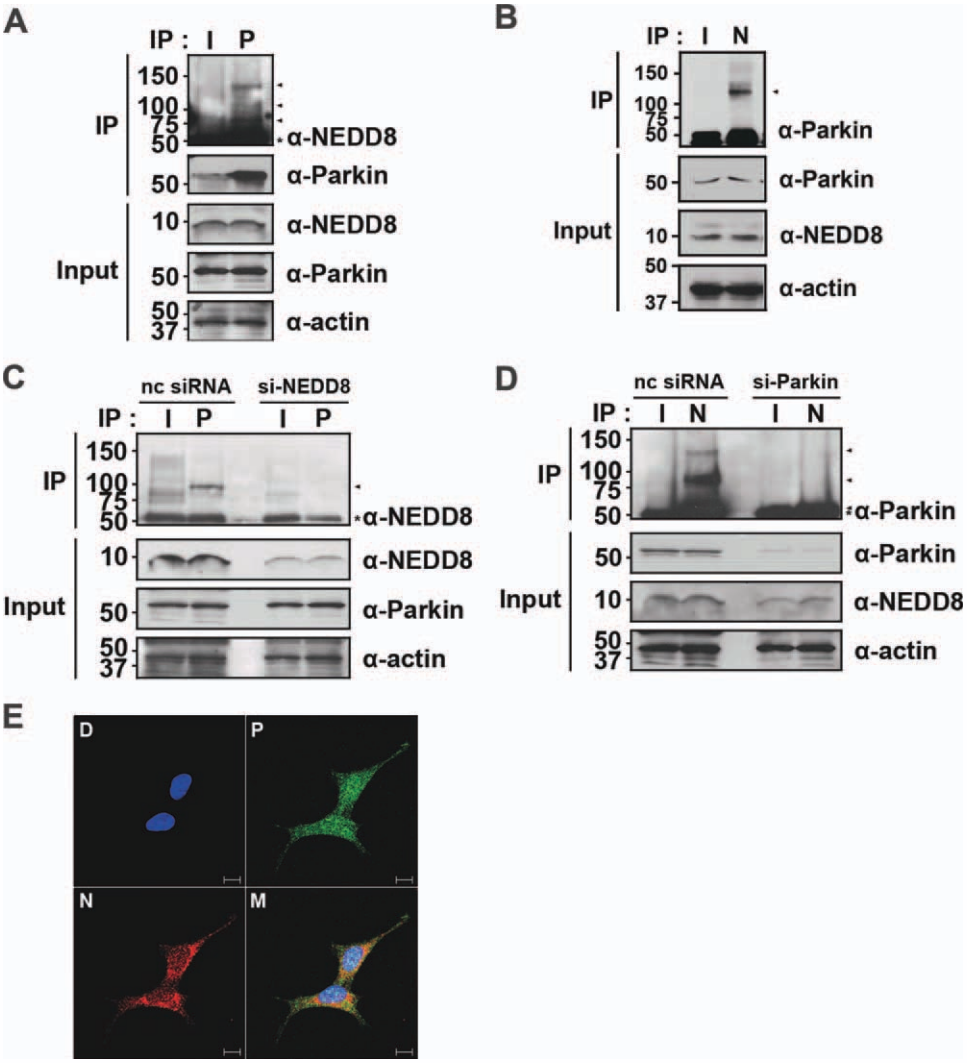

Fig. 2. Endogenous parkin is neddylated in SH-SY5Y cells. **A:** Coimmunoprecipitation/Western blot analysis of SH-SY5Y cell lysates. Immunoprecipitates obtained with anti-parkin (P) or preimmune IgG (I) as a control were probed with anti-NEDD8 or anti-parkin antibodies. Expression of endogenous proteins was confirmed by Western blot using anti-parkin or anti-NEDD8 antibodies. Actin served as a loading control. The arrowhead indicates neddylated parkin. **B:** Coimmunoprecipitation/Western blot analysis of SH-SY5Y cell lysates. Immunoprecipitates obtained with anti-NEDD8 (N) or preimmune IgG (I) as a control were probed with anti-parkin antibody. Expression of endogenous proteins was confirmed by Western blot using anti-NEDD8 or anti-parkin antibodies. **C,D:** SH-SY5Y cells were transfected with parkin siRNA (si-parkin; 200 nM; D), NEDD8 siRNA (si-NEDD8; 200 nM; C), or nonspecific control siRNA (nc siRNA; 200 nM). Lysates were immunoprecipitated with either anti-NEDD8 or preimmune IgGs (IgG) and immunoblotted with anti-parkin or anti-NEDD8 antibodies. Whole-cell lysates were immunoblotted with anti-parkin or anti-NEDD8 antibodies. **E:** Confocal images showing immunocytochemical staining for parkin (P) and NEDD8 (N) in SH-SY5Y neuroblastoma cells. Nuclei were counterstained with DAPI. These results are representative of three independent experiments. Scale bars = 10 μm.

amino acids 101–294. We tested other parkin mutants having single point mutations in regions outside of amino acid 101–294 (such as K27R, K32R, K48R, K76R, K349R, K369R, K408R, K413R, K427R, or K435R) and found that all these mutants underwent neddylation (Supp. Info. Fig. 3E,G). A parkin mutant harboring two point mutations at K412R and K416R was also modified with NEDD8 (Supp. Info. Fig. 3G), as was a PD-linked parkin mutant with K161N mutation (Supp. Info. Fig. 3F). These results, taken with the finding that multiple parkin bands are detected in the presence of NEDD8 (Fig. 1A,B), suggest that the parkin is neddylated at multiple sites along its entire length.

Furthermore, we examined whether several familial PD-linked parkin mutants (i.e., T240R, R334C, or C441R) could be neddylated. As shown in Supporting Information Figure 3H, they did not show any significant change of covalent neddylation pattern. These results suggest that at least these three mutants still become the target of neddylation.

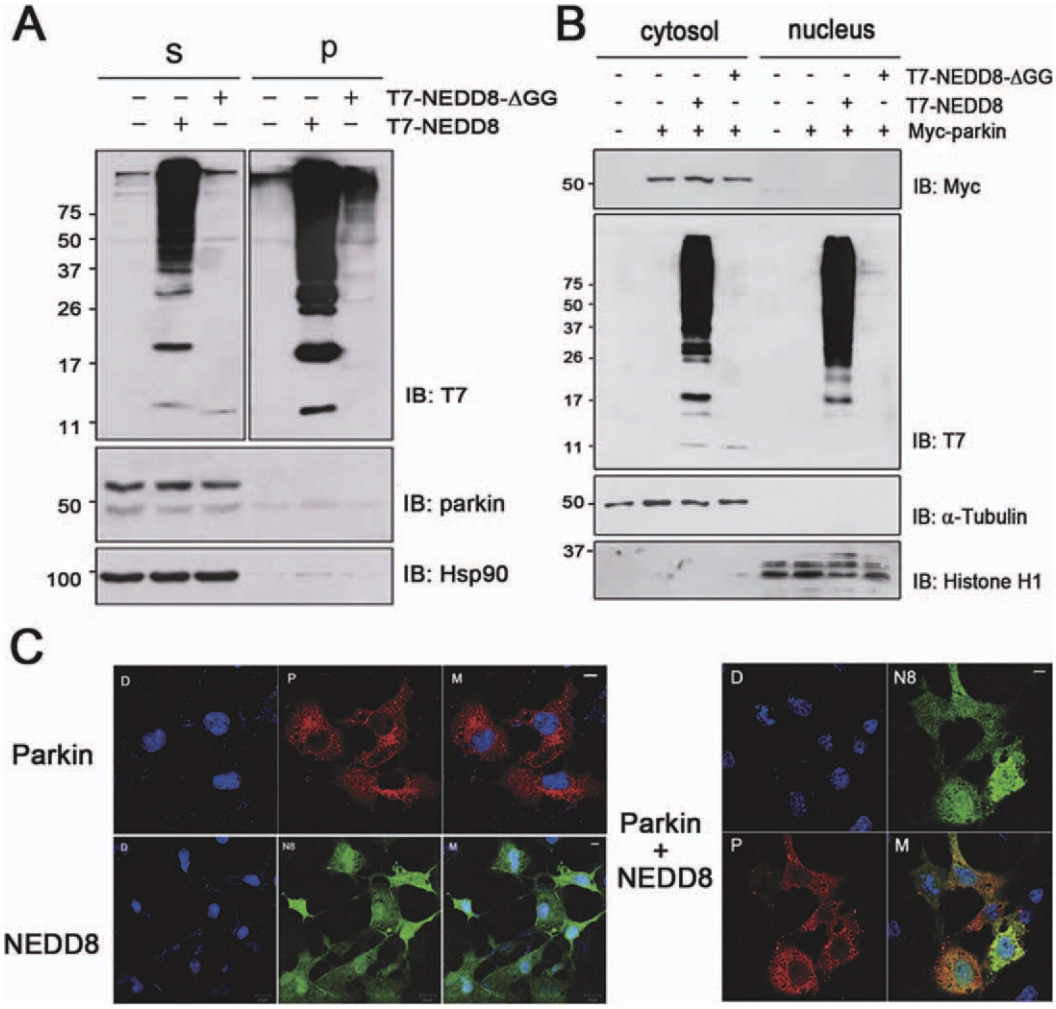

Fig. 3. NEDD8 does not affect the solubility or subcellular localization of parkin. **A:** HEK293 cells were transfected with plasmids encoding T7-tagged NEDD8 or NEDD8-ΔGG. Cells were lysed in buffer containing 1% NP-40 (S fraction) and 2% SDS (P fraction). The resulting fractions were subjected to Western blot analysis using anti-T7 or anti-parkin antibodies. Hsp90 served as a loading control. **B:** HEK293 cells were mock-transfected or transfected with plasmids encoding Myc-parkin, T7-tagged NEDD8, or T7-NEDD8-ΔGG. Cytosolic and nuclear fractions were then analyzed by Western blot using anti-Myc or anti-T7 antibodies. The purity of each fraction was confirmed by Western blot analysis of α-tubulin (cytosolic marker) or histone H1 (nuclear marker). **C:** Confocal images showing immunocytochemical staining for GFP-tagged NEDD8 (N8) and Myc-tagged parkin (P) in Cos7 cells. Cells were labeled and counterstained with DAPI at 24 hr after transfection with either factor alone (left) or in combination (right). These results are representative of three independent experiments. Scale bars = 10 μm.

Solubility and Subcellular Localization of Parkin Are Not Changed by NEDD8

To assess the functional consequences of parkin neddylation, we investigated whether NEDD8 attachment alters the solubility of parkin. We transfected HEK293 cells with T7-tagged NEDD8 or its dominant negative mutant (NEDD8-ΔGG) and then analyzed 1% NP-40-soluble and -insoluble fractions by Western blot using anti-parkin antibodies. No change in parkin solubility was detected among cells overexpressing wild-type NEDD8, mock-transfected control cells, or cells overexpressing NEDD8-ΔGG (Fig. 3A). We also investigated whether NEDD8 alters the subcellular localization of parkin by transfecting HEK293 cells with Myc-tagged parkin ± T7-tagged wild-type NEDD8 or T7-NEDD8-ΔGG. Western blot analysis of cytosolic and nuclear fractions from these cells revealed that parkin localization did not significantly differ before and after NEDD8 attachment (Fig. 3B). Moreover, confocal analysis showed that parkin was distributed evenly throughout the cytoplasm of Cos7 cells, irrespective of NEDD8 overexpression (Fig. 3C). These results indicate that NEDD8 modification does not affect the solubility or localization of parkin.

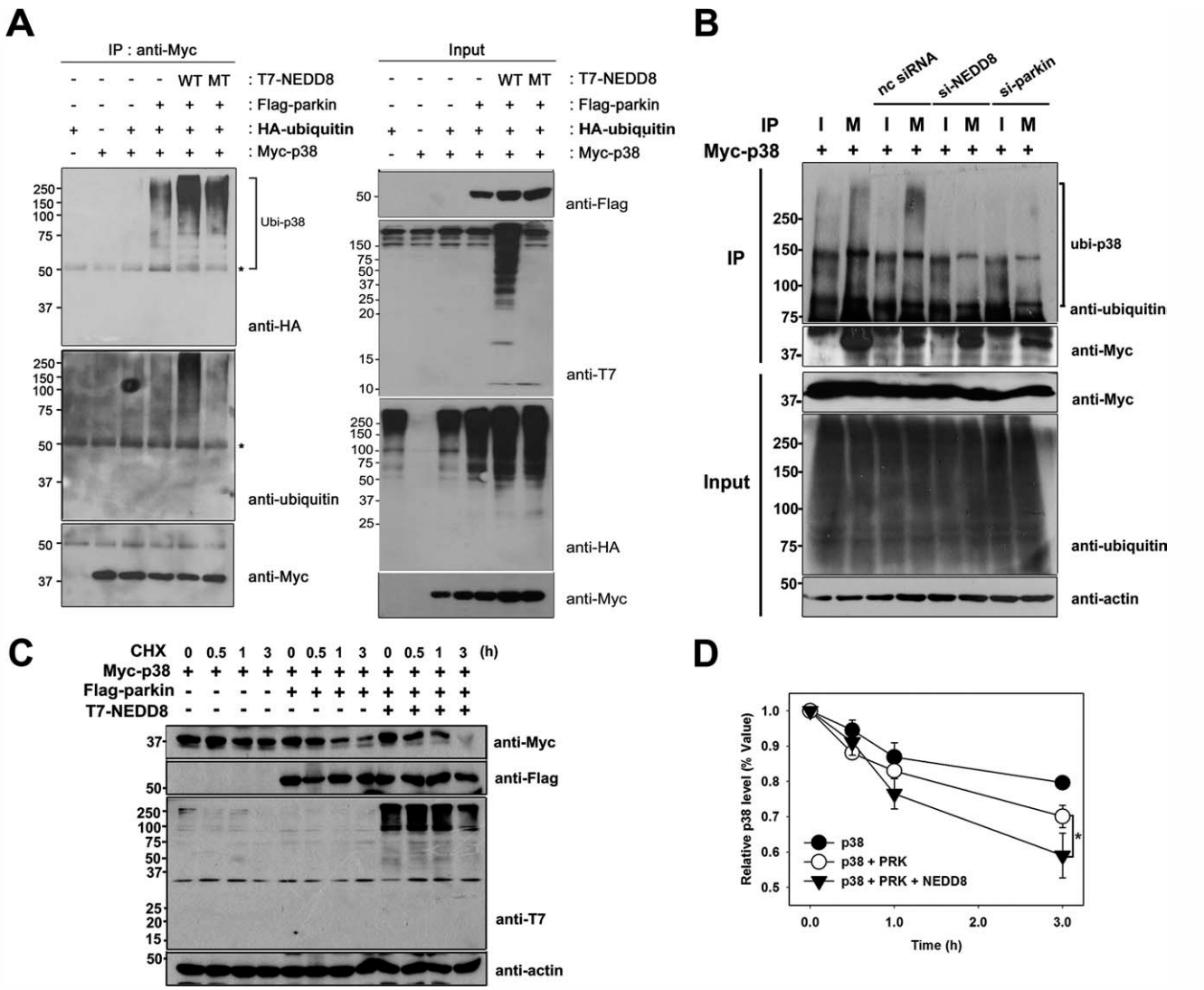

Fig. 4. The ubiquitin E3 ligase activity of parkin is enhanced by NEDD8. **A:** Effect of NEDD8 on parkin-mediated ubiquitination of p38. HEK293 cells expressing the indicated combinations of HA-tagged ubiquitin, Myc-tagged p38, Flag-tagged parkin, and T7-tagged NEDD8 (either wild-type [WT] or NEDD8 ΔGG [MT]) were incubated in the presence of the proteasomal inhibitor MG132 (10 μM) for 6 hr. Anti-Myc immunoprecipitates were then probed with anti-HA, anti-Myc, and anti-ubiquitin antibodies. Proper expression of tagged proteins was determined by Western blotting with anti-Flag, anti-T7, or anti-HA antibodies. **B:** SH-SY5Y cells were transfected with Myc-p38 alone or together with 200 nM of nonspecific control siRNA (nc siRNA) or siRNA against NEDD8 (si-NEDD8) or parkin (si-parkin). After 48 hr of incubation, cells were incubated in the presence of the proteasomal inhibitor MG132 (10 μM) for 6 hr. Lysates were immunoprecipitated with anti-Myc (M) or preimmune IgGs (I) and immunoblotted with anti-Myc or anti-ubiquitin antibodies. Proper expressions of exogenous p38 and endogenous ubiquitin were determined by Western blotting with anti-Myc, anti-ubiquitin antibodies. **C:** Effect of NEDD8 on the parkin-induced decrease in p38 stability. HEK293 cells were transfected with the indicated combination of Myc-tagged p38, Flag-tagged parkin, and T7-tagged NEDD8. They were then treated with 25 μg/ml cycloheximide for the indicated times. Whole-cell lysates were subjected to Western blot analysis using anti-Myc antibody. **D:** Relative p38 levels were quantified in Image J (NIH; $*P < 0.05$).

NEDD8 Promotes the Ubiquitin E3 Ligase Activity of Parkin by Enhancing the Binding Affinities for Its Ubiquitin-Conjugating E2 Enzyme and Substrates

Covalent attachment of NEDD8 to cullin1 enhances the E3 ligase activity of the SCF complex (Pan et al., 2004; Wu et al., 2006; Sakata et al., 2007). Therefore, we tested whether the E3 ligase activity of parkin is modulated by NEDD8. Parkin activity was measured by using in vivo ubiquitination assays in cultured cells. In these assays, the p38/JTV-1 subunit of the aminoacyl-tRNA synthetase complex served as the parkin substrate (Corti et al., 2003; Lim et al., 2007). Consistently with a previous report, p38 was well ubiquitinated by parkin (Fig. 4A). Interestingly,

parkin-mediated p38 ubiquitination was increased 1.7-fold by overexpression of wild-type NEDD8 but not by NEDD8-ΔGG (Fig. 4A), indicating that NEDD8 enhances the ubiquitin E3 ligase activity of parkin. When we tested whether NEDD8 directly binds to p38 as a negative control, co-IP assay revealed that p38 does not bind to NEDD8. Moreover, p38 was found not to be a target of covalent neddylation (Supp. Info. Fig. 5A). When we also examined the effect of NEDD8 on the ubiquitination of p38, in vivo ubiquitination assay showed that NEDD8 did not affect the ubiquitination of p38 in a remarkable way (Supp. Info. Fig. 5B). Furthermore, the immunoblot analysis of the cell lysates confirmed that NEDD8 transfection does not change p38 level significantly (Supp. Info. Fig. 5A,B). In addition, knockdown of endogenous NEDD8 or parkin by siRNA decreased the ubiquitination of p38 in SH-SY5Y cells, markedly in high-molecular-weight bands of p38 (Fig. 4B).

Next, we determined the effect of parkin on p38 half-life in the absence or presence of NEDD8. To do this, we analyzed p38 levels following cycloheximide treatment of HEK293 cells that were transfected with different combinations of plasmids encoding Myc-p38, Flag-parkin, or T7-NEDD8. As expected, cotransfection of only parkin and p38 was associated with considerably lower p38 stability than p38 transfection alone (Fig. 4C,D). When cells were transfected with NEDD8, parkin-mediated p38 degradation occurred much faster (Fig. 4C,D). This effect could be caused by increased enzymatic activity of parkin or enhanced stability of parkin in the presence of NEDD8.

To test the latter possibility, we measured the half-life of parkin in the presence or absence of NEDD8 after cycloheximide treatment. We found that parkin stability remained the same, regardless of the presence of NEDD8 (Supp. Info. Fig. 4). This suggests that NEDD8 enhances parkin-induced degradation of p38 level by increasing parkin enzymatic activity, not parkin stability. To understand how NEDD8 attachment may stimulate parkin enzymatic activity, we analyzed the effect of NEDD8 on the association between parkin and E2 ubiquitin-conjugating enzymes. Previous studies have shown that neddylation of E3 ubiquitin ligase accelerates the formation of a complex between ubiquitin-conjugating E2 enzyme and ubiquitin E3 ligase, stimulating polyubiquitination of the substrate (Kawakami et al., 2001; Sakata et al., 2007). Given that parkin interacts with the E2 ubiquitin-conjugating enzymes UbcH7 and UbcH8 (Imai et al., 2000; Shimura et al., 2000), we tested whether NEDD8 could recruit UbcH7 or UbcH8 to parkin. Cells were cotransfected with parkin and E2 ubiquitin-conjugating enzymes (UbcH7 or UbcH8) alone or together with NEDD8. Co-IP assay of lysates from these cells showed that parkin binds to UbcH8. In addition, an increased association of parkin with UbcH8 was found in the presence of wild-type NEDD8 but not with conjugation-defective NEDD8-ΔGG (Fig. 5A). Furthermore, knockdown of endogenous NEDD8 decreased the binding of endogenous parkin to UbcH8 in SH-SY5Y cells (Fig. 5B). However, compared with UbcH8, parkin did not directly bind to UbcH7, and NEDD8 attachment had no effect on the association of parkin with UbcH7 (Fig. 5A). Another possible mechanism by which NEDD8 attachment may stimulate parkin enzymatic activity is by enhancing the substrate recognition ability of parkin. Consistently with this idea, the binding affinity of parkin for p38 was significantly enhanced by wild-type NEDD8 but not by NEDD8-ΔGG (Fig. 6A,B). On the contrary, knockdown of NEDD8 reduced the binding of endogenous parkin to p38 (Fig. 6C). A similar effect was observed for the association between parkin and TRAF2, another parkin substrate (Henn et al., 2007). Interestingly, wild-type NEDD8, but not NEDD8-ΔGG, dramatically increased the association of parkin with TRAF2, which usually binds to parkin weakly (Fig. 6D). These results show that NEDD8 enhances the binding affinities between parkin and its target proteins as well as between parkin and its ubiquitin-conjugating E2 enzyme, UbcH8, effects that may underlie the ability of NEDD8 to stimulate parkin activity.

NEDD8 Attachment Potentiates the Cytoprotective Function of Parkin Against 1-Methyl-4-Phenylpyridinium Ion

Parkin protects cells against death induced by various toxic agents such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine, and rotenone (Hyun et al., 2005; Casarejos et al., 2006; Moore, 2006; Vercammen et al., 2006). To determine whether neddylation affects the cytoprotective activity of parkin, we measured the effect of wild-type NEDD8 or NEDD8-ΔGG on MPP^+ (a toxic metabolite of MPTP)-induced toxicity in either SH-SY5Y cell lines stably overexpressing parkin or parental SH-SY5Y cells. MPP^+ caused significant death in parental cells, reducing cell viability by approximately 30% (Fig. 7A). In contrast, cells stably overexpressing wild-type parkin showed a slight, albeit nonsignificant, increase in cell viability under these conditions. Importantly, transfection of wild-type NEDD8 significantly increased viability by ~20% in parkin-stable cells but not in parental controls (Fig. 7A). Unlike wild-type NEDD8, NEDD8-ΔGG was unable to increase viability in parkin-stable cells (Fig. 7A). Furthermore, the knockdown of NEDD8, parkin, or Ubc12 level by each siRNA significantly decreased cell viability against MPP^+ by ~40%, 10%, or 5%, respectively (Fig. 7B). The cytoprotective effect of parkin neddylation was not observed following rotenone exposure (Supp. Info. Fig. 6). These data show that neddylation promotes the ability of parkin to protect against MPP^+-induced cell death.

DISCUSSION

Parkin acts as an E3 ubiquitin ligase (Shimura et al., 2000), and several substrates have been identified. Given this function, one could surmise that loss of *parkin*

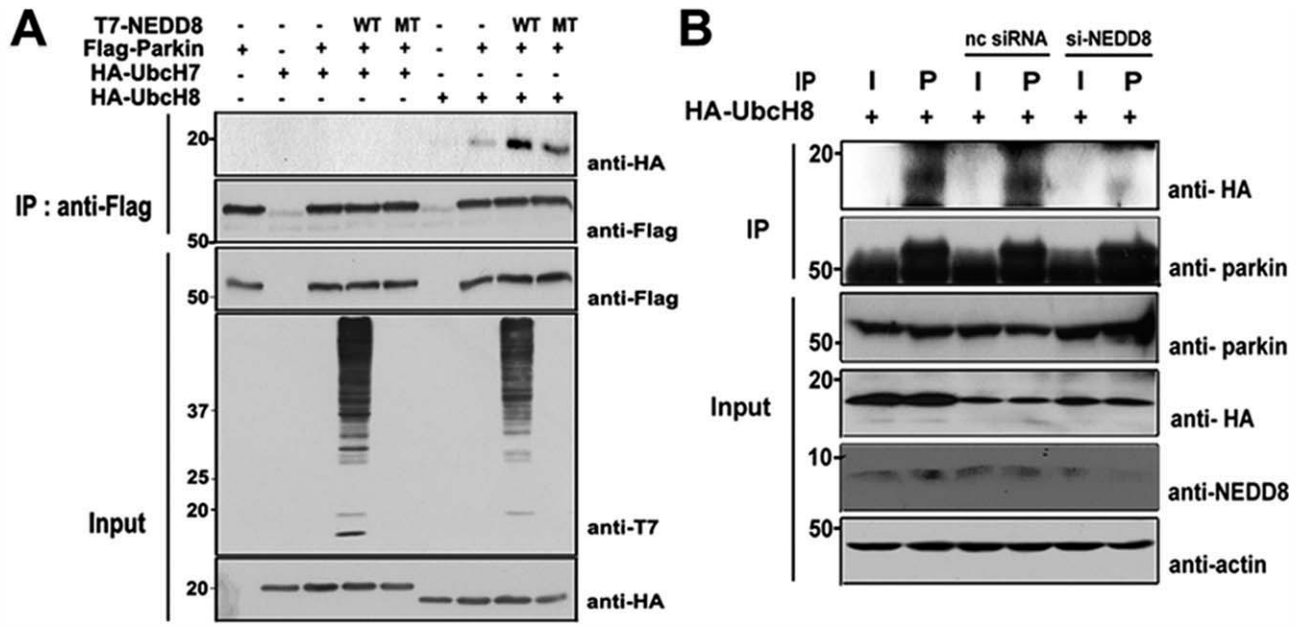

Fig. 5. Effect of NEDD8 attachment on the binding of parkin to UbcH7 and UbcH8. **A:** Where specified, HEK293 cells were transfected with the indicated combinations of HA-tagged UbcH7, HA-UbcH8, Flag-Parkin, and T7-tagged NEDD8 forms encoding either wild-type (WT) or conjugation-defective ΔGG (MT). Cells were lysed in buffer containing 1% NP-40, and anti-Flag coimmunoprecipitates were then subjected to Western blot analysis using anti-HA. Proper expression of the target proteins was determined by probing whole-cell lysates with anti-HA, anti-Flag, and anti-T7 antibodies. The blot is representative of three independent experiments. **B:** SH-SY5Y cells were transfected with HA-UbcH8 alone or together with 200 nM of either nonspecific control siRNA (nc siRNA) or siRNA against NEDD8 (si-NEDD8). Lysates were immunoprecipitated with anti-parkin (P) or preimmune IgGs (I) and immunoblotted with anti-HA or anti-parkin antibodies. Proper expressions of exogenous UbcH8 and endogenous parkin or NEDD8 were determined by Western blotting with anti-HA, anti-parkin, or anti-NEDD8.

activity results in gradual and abnormal accumulation of substrates, which then induce neuronal cell death and probably the development of familial PD syndrome. For this reason, understanding the molecular mechanisms underlying the regulation of parkin enzymatic activity is important. Studies have shown that the enzymatic activity and neuroprotective role of parkin are modulated by a number of posttranslational modifications and noncovalent interactions with other proteins. As is the case with the activity of most other enzymes, parkin activity is regulated primarily through phosphorylation. For example, casein kinase 1, protein kinase A, protein kinase C, and cyclin-dependent kinase 5 phosphorylate parkin, leading to a reduction in enzymatic activity (Yamamoto et al., 2005; Avraham et al., 2007). Parkin also undergoes S-nitrosylation, which has been shown to occur in PD and to inhibit parkin ubiquitin ligase activity (Chung et al., 2004; Yao et al., 2004). In addition, parkin is positively regulated by noncovalent SUMO-1 binding (Um and Chung, 2006) and negatively regulated by interacting with 14-3-3eta (Sato et al., 2006) or BAG5 (Kirkin and Dikic, 2007). Here, we report a novel mode of parkin modulation by NEDD8, a ubiquitin-like protein. Our data show that parkin becomes neddylated in cultured cell lines, leading to an increase in its ubiquitin E3 ligase activity.

Similar to other posttranslational modifications, neddylation causes the structural modification of target proteins and consequently alters their biochemical and functional properties, such as subcellular localization, protein interaction between binding partners, and/or enzymatic activity (Kirkin and Dikic, 2007; Rabut and Peter, 2008). For example, the neddylation of cullin stimulates the ubiquitin E3 ligase activity of SCF complexes (Sakata et al., 2007). In addition, NEDD8 modification of CUL1 dissociates CAND1, an inhibitor of CUL1-SKP1 binding and ligases (Liu et al., 2002). Furthermore, neddylation can promote the recruitment of NEDD8-modifying proteins (Rabut and Peter, 2008). For example, NEDD8 forms an active platform on the SCF complex for selective recruitment of the ubiquitin-conjugating E2 enzyme Ubc4 and thereby up-regulates the activity of cullin-based ubiquitin ligases (Sakata et al., 2007).

Here we found that NEDD8 did not appreciably affect the solubility or subcellular localization of parkin. Instead, NEDD8 increased parkin E3 ligase activity by enhancing the binding affinity of parkin to ubiquitin-conjugating E2 enzyme UbcH8 but not to UbcH7. In addition, NEDD8 attachment led to an enhanced interaction between parkin and substrates. Although the molecular mechanism underlying this effect remains to

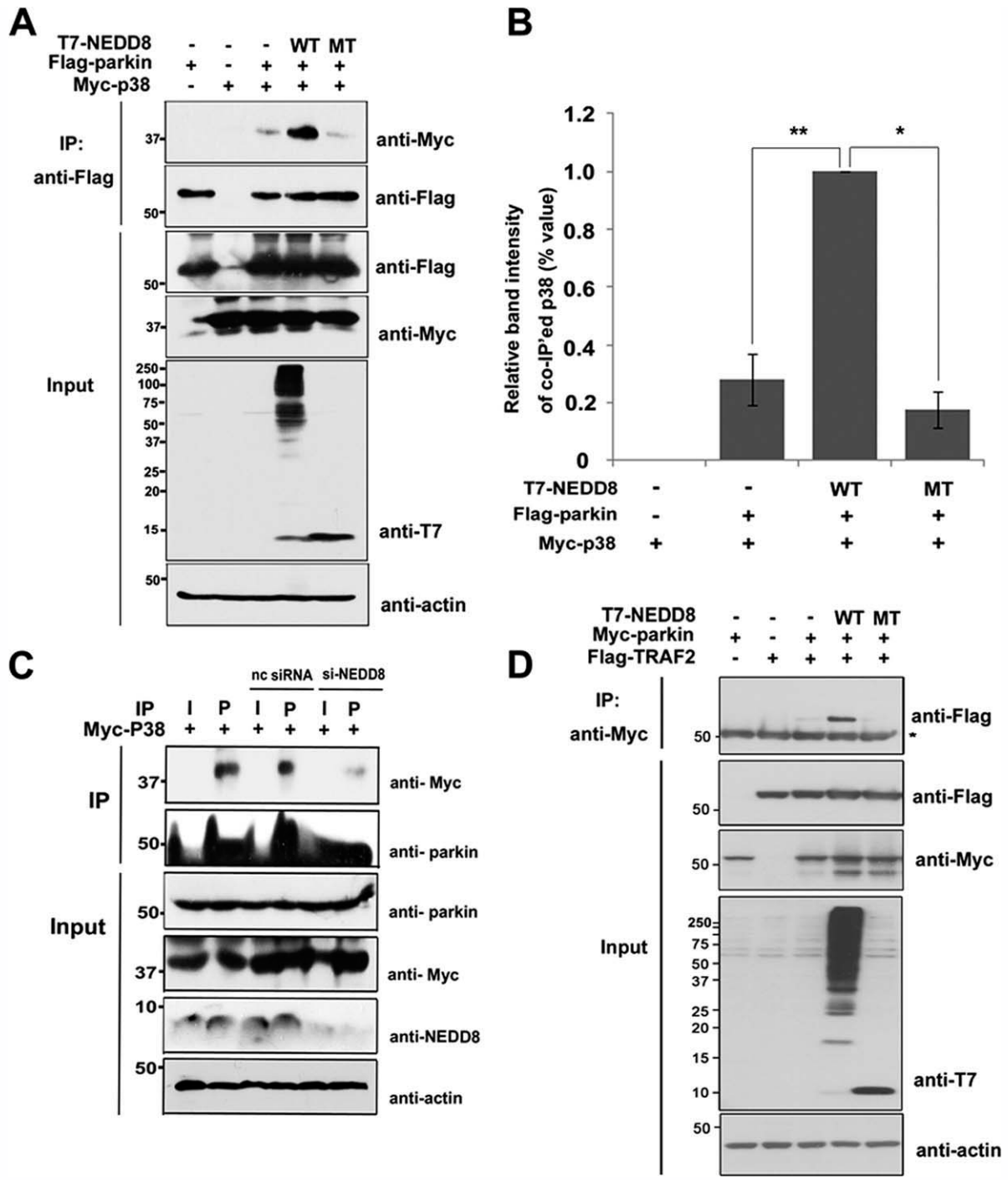

Fig. 6. NEDD8 enhances the ability of parkin to recognize its substrate. **A:** HEK293 cells overexpressing the indicated combinations of Myc-p38, Flag-parkin, and either T7-NEDD8 (WT) or T7-NEDD8-ΔGG (MT) were incubated in the presence of the proteasomal inhibitor MG132 (10 μM) for 3 hr. Anti-Flag immunoprecipitates were then probed with anti-Myc antibodies. Proper expression of tagged proteins was confirmed by Western blotting using anti-Myc, anti-Flag, and anti-T7 antibodies. Actin served as a loading control. **B:** Relative band intensities of Myc-p38 in each co-IP sample were quantified using MultiGauge V3.1. Linear graphs are the representative of three independent experiments ± range. $*P < 0.05$, $**P < 0.01$. **C:** SH-SY5Y cells were transfected with Myc-p38 alone or together with 200 nM of either nonspecific control siRNA (nc siRNA) or siRNA against NEDD8 (si-NEDD8). After 48 hr incubation, cells were incubated in the presence of the proteasomal inhibitor MG132 (10 μM) for 6 hr. Lysates were immunoprecipitated with anti-parkin (P) or preimmune IgGs (I) and immunoblotted with anti-Myc or anti-parkin antibodies. Proper expressions of exogenous p38 and endogenous parkin or NEDD8 were determined by Western blotting with anti-Myc, anti-parkin, or anti-NEDD8. **D:** HEK293 cells were transfected with Myc-parkin, Flag-TRAF2, and either T7-NEDD8 (WT) or T7-NEDD8-ΔGG (MT). Anti-Myc immunocomplexes were probed with anti-Flag antibodies. Proper expression of each protein was confirmed by Western blotting using anti-Myc, anti-Flag, and anti-T7 antibodies. The blots are the representative of at least three independent experiments.

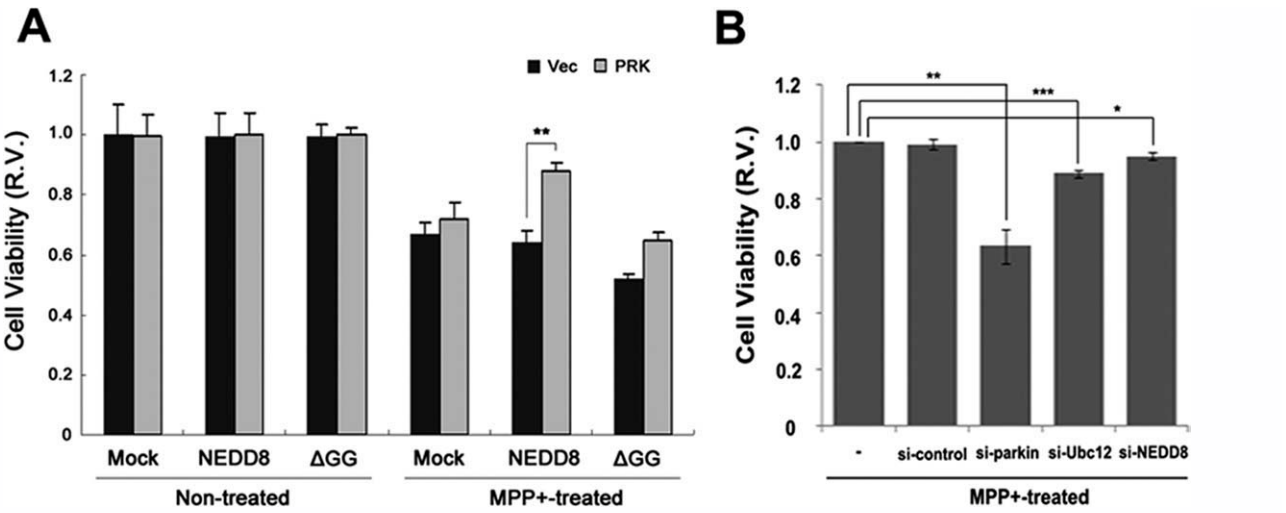

Fig. 7. NEDD8 enhances the neuroprotective activity of parkin. **A:** SH-SY5Y cells stably expressing parkin (PRK) or parental cells (vec) were mock transfected (Mock) or transfected with T7-NEDD8 or T7-NEDD8-ΔGG. Cells were then left untreated or were treated with 200 μM of MPP^+ for 24 hr. Viability was assessed by CCK-8 assay. Data are expressed as the mean of three independent experiments (**$P < 0.01$). **B:** SH-SY5Y cells stably expressing parkin (PRK) were transfected with T7-NEDD8 alone or together with 200 nM of either nonspecific control siRNA or siRNA against NEDD8, parkin, or Ubc12, as indicated. After 72 hr incubation, cells were left untreated or treated with 200 μM of MPP^+ for 24 hr. Viability was assessed by CCK-8 assay. Data are expressed as the mean of three independent experiments. *$P < 0.005$, **$P < 0.001$, ***$P < 0.0005$.

be elucidated, it is possible that covalent modification of parkin with NEDD8 induces conformational changes that favor the binding of parkin to substrates and other proteins.

Neddylated proteins can be divided into two groups based on whether they have a single conserved lysine residue that is neddylated (such as cullin and pVHL) or multiple neddylated residues (such as p53, BCA3, EGFR, and APP; Xirodimas, 2008). Our screening for parkin neddylation sites revealed that parkin is not neddylated at a single amino acid. Instead, neddylation appears to occur at the multiple sites along the parkin molecule. More exhaustive experiments will be necessary to define the complete pattern of parkin neddylation and its functional consequences.

The NEDD8 E3 ligase for many neddylation targets has yet to be identified. Nevertheless, several enzymes have been found to mediate the neddylation of some targets directly. These include Rbx1 and/or Dcn1 for the modification of cullin, Parc, and Cul7; Mdm2 for p53, p73, and Mdm2 itself; and c-Cbl for EFGR (Rabut and Peter, 2008). MDM2, a well known ubiquitin E3 ligase of p53, has NEDD8 E3 ligase activity and becomes autoneddylated (Xirodimas et al., 2004; Watson et al., 2006; Watson and Irwin, 2006).

Parkin and MDM2 both act as an ubiquitin E3 ligase, so their modifications by NEDD8 may occur through a similar process. Because MDM2 binds to Ubc12, an NEDD8-E2 enzyme during the neddylation process, we additionally investigated whether parkin directly binds to Ubc12. As with Myc-tagged MDM2 as a positive control, which was confirmed to bind to GST-Ubc12 (Supp. Info. Fig. 7A), in vitro GST pull-down assay revealed that parkin also binds to Ubc12 (Supp. Info. Fig. 7B). This finding suggests that parkin neddylation might occur in a manner similar to MDM2 neddylation. Interestingly, several neddylated proteins seem to be either substrates or components of ubiquitin E3 ligases, pointing to an intriguing relationship between ubiquitination and neddylation (Xirodimas, 2008), or this reflects only that parkin as a substrate binds to NEDD8-E2 enzyme for the covalent modification to proceed, and there seems to be no requirement of NEDD8-E3 enzyme in this reaction. However, an additional in vitro neddylation assay revealed that parkin is not neddylated in the presence of recombinant E1 and E2 (Supp. Info. Fig. 8). This finding indicates that parkin itself does not have a NEDD8 E3 ligase activity, or a critical factor(s) would be required for the parkin neddylation without the need of E3 enzyme. Identification of a suitable NEDD8 E3 ligase or additional factors for parkin neddylation requires further study.

NEDD8 has been observed in inclusion bodies in several neurodegenerative disorders, including PD (Dil Kuazi et al., 2003; Mori et al., 2005). Parkin and α-synuclein have been shown to colocalize in LBs (Schlossmacher et al., 2002). Whether LBs are cytotoxic or cytoprotective is controversial. However, recent studies indicate that LBs are cytoprotective proteinaceous inclusions formed at the centrosome that segregate and facilitate the degradation of excess amounts of unwanted and possibly cytotoxic proteins (Olanow et al., 2004). In this context, our current findings suggest that neddylated parkin could be recruited into aggresomes like LBs and

ubiquitinate misfolded proteins in these inclusions. Although further functional studies are necessary to link parkin neddylation to the pathogenesis of PD, this study provides evidence of a link between NEDD8 and PD pathogenesis.

ACKNOWLEDGMENTS

We thank K. Tanaka, G. Lee, H. Rhim, M. Ohh, C.Y. Choi, and S. Kim for providing plasmids and Y.J. Oh and W.T. Kim for valuable comments.

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