Parkin Phosphorylation and Modulation of Its E3 Ubiquitin Ligase Activity

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Ayako Yamamoto‡, Arno Friedlein§, Yuzuru Imai¶, Ryosuke Takahashi†, Philipp J. Kahle‡‡, and Christian Haass‡‡

From the ‡Laboratory of Alzheimer’s and Parkinson’s Disease Research, Department of Metabolic Biochemistry, Ludwig Maximilians University, Munich, Germany, §Roche Center for Medical Genomics, F. Hoffmann-La Roche Ltd. 4070 Basel, Switzerland, and ¶Motor System Neurodegeneration, RIKEN Brain Science Institute, Saitama 351-0198, Japan

Mutations in the PARKIN gene are the most common cause of hereditary parkinsonism. The parkin protein comprises an N-terminal ubiquitin-like domain, a linker region containing caspase cleavage sites, a unique domain in the central portion, and a special zinc finger configuration termed RING-IBR-RING. Parkin has an E3 ubiquitin-protein ligase activity and is believed to mediate proteasomal degradation of aggregation-prone proteins. Whereas the effects of mutations on the structure and function of parkin have been intensively studied, post-translational modifications of parkin and the regulation of its enzymatic activity are poorly understood. Here we report that parkin is phosphorylated both in human embryonic kidney HEK293 cells and human neuroblasta SH-SY5Y cells. The turnover of parkin phosphorylation was rapid, because inhibition of phosphatases with okadaic acid was necessary to stabilize phosphoparkin. Phosphoamino acid analysis revealed that phosphorylation occurred mainly on serine residues under these conditions. At least five phosphorylation sites were identified, including Ser101, Ser131, and Ser136 (located in the linker region) as well as Ser296 and Ser378 (located in the RING-IBR-RING motif). Casein kinase-1, protein kinase A, and protein kinase C phosphorylated parkin in vitro, and inhibition of casein kinase-1 caused a dramatic reduction of parkin phosphorylation in cell lysates. Induction of proteasome stress in cells reduced parkin phosphorylation, and unphosphorylated parkin had slightly but significantly elevated autoubiquitination activity. Thus, complex regulation of the phosphorylation state of parkin may contribute to the unfolded protein response in stressed cells.

Parkinson’s disease (PD) is the second most common neurodegenerative disorder. Parkinsonian symptoms are caused by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (1). Although more than 90% of PD cases occur sporadically, the study of genetic mutations has offered great insight into the molecular mechanisms of PD (2). After the discovery that mutations in the PARKIN gene cause autosomal recessive juvenile parkinsonism (3), parkin mutations have been recognized as the most common cause of hereditary PD and possibly a risk factor for idiopathic PD (4, 5).

The PARKIN gene comprises 12 exons and codes for a 465-amino acid protein that is widely expressed, most prominently in muscle and throughout the brain (3). The 52-kDa parkin protein comprises an N-terminal ubiquitin-like domain (aa 1–76), a unique parkin domain (aa 145–232), and two RING (really interesting new gene) fingers (aa 238–293 and 418–449, respectively) flanking an IER (in-between RING) domain (aa 314–377) at the C terminus. All of these domains appear to be functionally important, because PD mutations cluster in them (6).

Parkin functions in the ubiquitin-proteasome system as an E3 ubiquitin-protein ligase together with the E2 ubiquitin-conjugating co-enzymes UbcH7 or UbcH8 (7–9). Because this function appears to be defective in patients with parkin mutations, the identification of protein substrates is of great importance (6, 10). Parkin substrates include synaptic proteins (the septicins CDCrel-1 and CDCrel-2, synaptotagmin XI, and the septins CDCrel-1 and CDCrel-2), cyclin E, α/β tubulin, and the p38 subunit of the aminocacyl-tRNA synthetase complex (9, 11–17). Parkin expression was found to be neuroprotective in Drosophila (18) and in cell culture models of dopamine neuron loss (19–22). However, the exact molecular mechanisms of how parkin dysfunction causes PD remain to be elucidated (6, 10).

Post-translational modifications often regulate enzymatic activity. Nitrosylation of parkin was recently found to occur in PD, leading to an inhibition of its ubiquitin ligase activity (23, 24). Here we addressed the question whether phosphorylation of parkin occurred and, if so, by which kinases under what cellular conditions. Parkin was found to be phosphorylated on at least five serine residues. Casein kinase-1 (CK-1), protein kinase A (PKA) and protein kinase C (PKC) were identified as parkin kinases in vitro, and inhibition of CK-1 suppressed phosphorylation of parkin in cell lysates. Unfolded protein stress mediated by proteasomal inhibition or endoplasmic reticulum (ER) stress, but not oxidative stress, reduced the overall phosphorylation of parkin. Unphosphorylated parkin isolated from eukaryotic cells or purified as recombinant fusion protein from bacteria showed a small but significant increase of autoubiquitin ligase activity, compared with parkin phosphorylated in vivo and in vitro. Thus, we suggest that modulation of the phosphorylation state of parkin has a regulatory role on its E3 ubiquitin ligase activity.
Materials and Methods

Cell Culture, Transfection, and Establishment of Stable Transfectants—HEK293, HEK293T, and SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium with Glutamax (PAA Laboratories GmbH) supplemented with 10% fetal calf serum for HEK293 cells and HEK293T cells, and 15% for SH-SY5Y cells. Cells were transfected using Lipofectamine 2000 (Invitrogen) or FuGene (Roche Applied Science) according to the supplier’s instructions. Stable HEK293 and SH-SY5Y transfecants were selected with 200 or 22.5 μg/ml zeocin, respectively.

Construction of cDNAs—Human full-length parkin was amplified by PCR (all primer sequences are available upon request) using a parkin cDNA (a gift from R. Baumeister) and cloned into the XbaI/HindIII restriction sites of pcDNA3.1 neo− (Invitrogen), yielding MYC-parkin.

An additional C-terminal V5 tag was introduced by subcloning into pcDNA6/V5-His (Invitrogen) (MYC-parkin-V5). For mapping the phosphorylation sites, parkin fragments comprising the N terminus (aa 2–144), the N terminus and middle portion (aa 2–293), and the C terminus (aa 294–465) were generated by PCR using the appropriate oligonucleotide primers and cloned into the XbaI/HindIII restriction sites of pcDNA3.1 neo− or the NheI/EcoRI restriction enzyme sites of pcDNA6/V5-His, yielding the constructs MYC-parN(-V5), MYC-parN-V5, and MYC-parC-V5 in two-step PCR.

GST-parkin, GST-parN, and GST-parC were constructed by PCR and cloned into the EcoRI/NotI restriction sites of pGEX-4T-1 (Amersham Biosciences). Serine-to-alanine mutants were generated by mutating respective codons in GST-parN or GST-parC by two-step PCR.

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G-terminal FLAG-tagged parkin (FLAG-parkin) and C-terminal FLAG-tagged PaelR (PaelR-FLAG) were described elsewhere (8, 11).

RT-PCR—Total RNA was isolated from both HEK293 cells and stable HEK293 transfecants of parkin under various stresses usingpeqGOLD RNA Pure (Peqlab). First-strand cDNA was synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was performed using Taq DNA polymerase (Peqlab) and the following primers: BiP forward primer, 5′-CGCCATCCGCGGTC-3′; BiP reverse primer, 5′-GGCTCCTGTATGATCC-3′.

Immunoprecipitation and Immunoblotting—Cells were harvested 24 h after the transfection and lysed in buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) with proteinase inhibitors (Sigma) on ice for 15 min. Cell lysates were centrifuged at 4 °C at 16,000 × g for 20 min. Immunoprecipitations were carried out using protein G-Sepharose (Amersham Biosciences) or protein A-Sepharose, anti-Myc-agarose, anti-FLAG M2-agarose affinity gel (all from Sigma) on ice for 1 h. Immunoprecipitates were washed with lysis buffer three times (or, in the case of radiolabeled samples, six times). Western blots were probed with biotinylated antibodies against Grp78 and Grp94 (Stressgen), polyclonal anti-parkin (Cell Signaling), monoclonal anti-KDEL against glucose-regulated proteins (Stressgen), and PRK8 monoclonal anti-parkin (25) (kindly provided by V. Lee). Immunoprecipitates were separated by SDS-PAGE and transferred onto PVDF membrane (Immobilon; Millipore Corp.). Enhanced chemiluminescence detection reagents (Amersham Biosciences) were used to detect immunoblot signals of the following antibodies: 9E10 monoclonal anti-Myc (Developmental Studies Hybridoma Bank, University of Iowa), monoclonal anti-V5 (Invitrogen), monoclonal anti-KDEL against glucose-regulated proteins (Stressgen), polyclonal anti-parkin (Cell Signaling), and PRK8 monoclonal anti-parkin (25) (kindly provided by V. Lee).

In Vivo Phosphorylation Assay—Twenty-four h after the transfection, cells were incubated for 45 min in phosphate-free medium (Sigma), and 13–36 MBq of [32P]orthophosphate was added. After 30 min (to label transfected parkin) or 2 h (to label endogenous parkin), 1 μM okadaic acid (OA) was added and incubated at 37 °C for 1 h. The conditioned medium was aspirated, and the cell monolayer washed twice with ice-cold phosphate-buffered saline. Cells were lysed on ice with lysis buffer, and immunoprecipitations were performed as above. Immunoprecipitates were separated by SDS-PAGE and transferred onto PVDF membrane. Autoradiography was carried out to visualize radiolabeled proteins.

Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry—Phosphorylation of transfected MYC-parkin fragments was induced with OA, and MYC immunoprecipitates were subjected to SDS-PAGE. Colloidal blue-stained bands of interest were in-gel digested with endoproteinase Lys-C as described (26). After overnight digestion, about 1 μl was mixed with 1 μl of saturated α-cyanocinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid in water and applied to the MALDI target. The samples were analyzed with a Bruker Daltonics (Bremen, Germany) Ultraflex TOF/TOF mass spectrometer.

An acceleration voltage of 25 kV was used. Calibration was internal to the samples with des-Arg-bradykinin and ACTH-(18–38) (both peptides purchased from Sigma).

Nanoelectrospray Ionization Tandem Mass Spectrometry—In order to identify which of the three possible serines was phosphorylated in the parkin peptide obtained after digestion with endoproteinase Lys-C with a monoisotopic mass of 1588.75 Da, this proteolytic product was subjected to nanoelectrospray ionization tandem mass spectrometry on a QSTAR Pulsar I quadrupole TOF tandem mass spectrometer (Applied Biosystems/MDS-Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (Proxen, Odense, Denmark) as described (27).

Dephosphorylation by Alkaline Phosphatase—HEK293 cells and SH-SY5Y cells were transiently transfected with MYC-parkin-V5 and various portions of parkin (MYC-parN-V5, MYC-parN-M-V5, and MYC-parC-V5). Twenty-four h after the transfection, 1 μM OA was added, and cells were incubated for 1 h. Immunoprecipitation using anti-Myc-agarose conjugate (Sigma) was performed. Immunoprecipitates were incubated at 37 °C for 1 h with or without calf intestinal alkaline phosphatase (CIP) (New England Biolabs) according to the supplier’s manual. Reactions were terminated by adding 2× SDS sample buffer and analyzed by immunoblot.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed using the method by Jelinek and Weber (28). After electroblotting radiolabeled proteins onto PVDF membrane, bands were excised and hydrolyzed using 6 N HCl at 100 °C for 90 min. After centrifugation, supernatants were dried in a SpeedVac concentrator. Pellets were dissolved in pH 2.5 buffer (5.9% glacial acetic acid, 0.8% formic acid, 0.3% pyridine, and 0.3 mM EDTA) and spotted onto thin layer chromatography plates (Merck) together with unlabeled phosphoamino acid markers (1 μg each of Ser(P), Thr(P), and Tyr(P); Sigma). One-dimensional high voltage electrophoresis was performed at 20 mA for 50 min. Radioactive phosphoamino acids were identified by autoradiography and co-migration with the ninhydrin-stained standards.

In Vitro Phosphorylation Assays—Recombinant rat CK-1 δ, recombinant α-subunit of human CK-2 and recombinant human Akt1/PKB protein kinase were used for in vitro phosphorylation assays according to the supplier’s instructions (Cell Signaling). The catalytic subunit of PKA purified from bovine heart (gift from V. Kinzel) was used in a similar buffer to PKA supplemented with 1 μM phorbol-12,13-dibutyrate (DMSO), 0.5 mM CaCl2, and 100 μM/ml phosphatidyserine under mixed micellar conditions. Fusion proteins GST-parkin, GST-parN, GST-parC, and various serine-to-alanine mutations in GST-parN or GST-parC were used as substrates. The reaction was started by adding 10 μM [γ-32P]ATP (250 μM [γ-32P]ATP in the case of Akt1/PKB) and allowed to proceed for 30 min at 30 °C.

Fig. 1. Parkin is phosphorylated both in HEK293 cells and SH-SY5Y cells. A, HEK293 cells (upper panels) and SH-SY5Y cells (lower panels) stably expressing MYC-parkin were labeled with [32P]orthophosphate in the presence or absence of OA, and then cell lysates were immunoprecipitated with 9E10. Proteins separated by 12% SDS-PAGE were transferred onto PVDF membrane. Autoradiography was carried out in order to visualize phosphorylated parkin (left panels). Afterward, blots were probed with polyclonal anti-parkin or 9E10 anti-Myc (right panels). B, endogenous parkin in HEK293 cells (upper panels) and SH-SY5Y cells (lower panels) was labeled with [32P]orthophosphate and immunoprecipitated with polyclonal anti-parkin. After the autoradiography (left panels), blots were probed with PRK8 monoclonal anti-parkin (right panels). DMSO, Me2SO.
Parkin Is Constitutively Phosphorylated at the N Terminus and the C Terminus—

In order to examine whether or not parkin is phosphorylated, we carried out in vitro phosphorylation assays. Stable HEK293 and SH-SY5Y transfectants expressing MYC-parkin were labeled with \[^{32}P\]orthophosphate. Phosphorylation was stabilized with OA, which inhibits phosphoprotein phosphatase 1, 2A, and 2B. Analysis of Myc-immunoprecipitated parkin by autoradiography revealed that full-length MYC-parkin undergoes phosphorylation both in HEK293 cells and SH-SY5Y cells (Fig. 1A). OA treatment also increased the \[^{32}P\]orthophosphate incorporation into endogenous parkin present at low levels in HEK293 and SH-SY5Y cells (Fig. 1B).

To assess which portion of parkin is phosphorylated, we generated Myc-tagged N-terminal parkin (aa 2–144) (parN) and C-terminal parkin (aa 294–465) (parC) constructs (Fig. 2A) and established their stable transfectants. Stable transfectants from the middle portion of parkin (aa 145–293) could not be established, possibly due to folding difficulties of the polypeptide. Both N-terminal and C-terminal parkin fragments were phosphorylated (Fig. 2B). We also noted in the anti-Myc immunoprecipitates phosphorylated protein bands that were possibly cleavage products derived from full-length parkin (29–31) and resembled the parN fragments in terms of \[^{32}P\]O\(_4\) incorporation and band shift. Retarded electrophoretic motility of the phosphorylated bands was evident for N-terminal parkin fragments, whereas such a mobility shift was not observed in parC (Fig. 2B). Thus, parkin is phosphorylated in both N terminus (with mobility shift) and C terminus (without mobility shift).

In order to further prove that parkin is phosphorylated and the observed electrophoretic motility shift of parN is caused by the covalent incorporation of phosphate, parkin immunoprecipitates were dephosphorylated with CIP. For this set of experiments, HEK293 and SH-SY5Y cells were transiently transfected with double-tagged MYC-parkin-V5, MYC-parN-V5, and MYC-parC-V5 (Fig. 3A). Transiently transfected cells were treated with OA and lysed, and the washed Myc-agarose immunoprecipitates were incubated with or without CIP. Then samples were subjected to 10–15% Tris/glycine gel electro-
phoresis, and immunoblots were probed with anti-V5 antibody. MYC-parN-V5 immunoprecipitated from OA-treated cells showed retarded electrophoretic mobility compared with controls (Fig. 3B). Full-length MYC-parkin-V5 also showed slight band retardation after OA treatment. These phosphorylation-induced band shifts were observed both in HEK293 and SH-SY5Y cells, indicating similar phosphorylation patterns in non-neuronal neuronal cells. The phosphorylation-induced band retardations were reversed upon CIP treatment (Fig. 3B), confirming that phosphorylation in the N terminus of parkin occurs along with a characteristic band shift. Electrophoretic mobility shift caused by the incorporation of covalent phosphate is frequently observed in phosphorylated proteins (e.g. tau (32) and the C-terminal fragment of presenilin-1 (33)). On the other hand, band shifts were hardly observed in parNM and parC, despite the fact that parC was phosphorylated in vivo (Fig. 2). Note that a band shift is not always a consequence of phosphorylation (34).

**Fig. 3.** Dephosphorylation reverses the molecular mass shift of N-terminal parkin both in HEK293 cells and SH-SY5Y cells. A, schematic representation of MYC-parkin-V5, MYC-parN-V5, MYC-parNM-V5, and MYC-parC-V5. B, the indicated constructs were transiently transfected into HEK293 cells (left panels) and SH-SY5Y cells (right panels). Twenty-four h after the transfection, 1 μM OA (lanes 2, 3, 5, 6, 8, 9, 11, and 12) or Me2SO (lanes 1, 4, 7, and 10) was added and cultured for 2 h. Immunoprecipitation (IP) was carried out using MYC-agarose beads. Immunoprecipitates were incubated in the presence (lanes 3, 6, 9, and 12) or absence (lanes 1, 2, 4, 5, 7, 8, 10, and 11) of CIP. Samples were subjected to 10–20% Tris-glycine gel (Invitrogen) or 15% SDS-PAGE. *: unspecific band. The band shifts of full-length parkin from SH-SY5Y cells are better seen on a short exposure (inset). C, parkin expression levels in each transient transfectant treated with Me2SO (lanes 1, 3, 5, and 7) or OA (lanes 2, 4, 6, and 8) were confirmed by immunoblotting (IB) of cell lysates using anti-V5 antibody (lower panels).
Identification of Parkin Phosphorylation Sites—To identify the phosphorylated residues of parkin in OA-treated cells, phosphoamino acid analysis was carried out. Stable transfectants of MYC-parkin in HEK293 cells and SH-SY5Y cells as well as MYC-parN and MYC-parC in HEK293 cells were labeled with \(^{32}P\)orthophosphate in the presence of OA. Myc immunoprecipitates were subjected to SDS-PAGE and then transferred onto PVDF. Proteins were eluted from the excised bands and hydrolyzed. Phosphoamino acid analysis revealed that parkin was mainly phosphorylated at serine residues in OA-treated HEK293 cells and SH-SY5Y cells (Fig. 4). Some minor threonine phosphorylation was observed, whereas tyrosine phosphorylation was not evident under these conditions.

There are 30 serine residues in parkin, 14 of them in parN and 4 in parC. We carried out site-directed mutagenesis to identify phosphorylated serine sites. Selected serines with high phosphorylation probability identified with the NetPhos 2.0 prediction algorithm were substituted by alanine in order to generate unphosphorylatable forms. Since there are only 4 serines in parC (Ser\(^{296}\), Ser\(^{378}\), Ser\(^{384}\), and Ser\(^{407}\)), we mutated all of them. These serine-to-alanine mutants in parN and parC as well as wild type with Myc tag at the N terminus and V5 tag at the C terminus were transiently transfected into HEK293 cells, and then cells were labeled with \(^{32}P\)orthophosphate in the presence or absence of OA. Cell lysates were subjected to immunoprecipitation using Myc-agarose beads. Phosphate incorporation of S101A, S131A, and S136A mutants under the treatment of OA were reduced compared with wild type parN (Fig. 5A). In the case of S101AlparN, the shifted band was no longer detected. Thus, Ser\(^{101}\) was found to be the responsible phosphate acceptor for the motility shift of parN. \(^{32}P\)PO\(_4\) incorporation into the lower band was detectable but reduced for the S101A, S131A, and S136A mutants of parN.

We also carried out mass spectrometry to determine phosphorylation sites in parN and confirmed that Ser\(^{131}\) and Ser\(^{136}\) (Fig. 5, B and C) were unambiguously phosphorylated, with weaker signals for Ser\(^{131}\) than for Ser\(^{136}\). The phosphorylation of Ser\(^{101}\) was not detected by mass spectrometry analysis, since Lys-C proteolysis of parN could not provide complete coverage of the protein sequence.

In the case of parC, S296A was the only mutant that revealed a small but reproducible reduction of phosphate incorporation compared with wild type parC in the presence of OA (Fig. 5D). No difference of phosphate incorporation between wild type parC and mutants S384A and S407A was detected. However, Ser\(^{378}\) was clearly identified as phosphorylation site using an alternative assay (see below). Taken together, we discovered that Ser\(^{101}\), Ser\(^{131}\), and Ser\(^{136}\) in the parkin N terminus as well as Ser\(^{296}\) (Fig. 5) and Ser\(^{378}\) (see Fig. 7B) in the parkin C terminus are phosphorylated.

Parkin Is Phosphorylated by CK-1, PKA, and PKC—In vitro phosphorylation assays were carried out in order to identify kinases involved in phosphorylation of parkin. Fusion proteins GST-parkin, GST-parN, and GST-parC as well as mutations GST-[S101A]parN, GST-[S131A]parN, GST-[S136A]parN, GST-[S296A]parC, and GST-[S378A]parC were expressed in *Escherichia coli*. The recombinant parkin fusion proteins were incubated in the presence of \([\gamma-\text{P}]\text{ATP}\) with various protein kinases. CK-1, CK-2, PKA, PKC were chosen because several recognition consensus sites for CK-1, PKA, and PKC were predicted by NetPhos 2.0 algorithm. The antiapoptotic kinase Akt/PKB1 was also considered, because parkin has been reported to protect dopaminergic cells against apoptosis (19–22).

We analyzed further which kinases were responsible for selective phosphorylation of residues Ser\(^{101}\), Ser\(^{131}\), Ser\(^{136}\), Ser\(^{296}\), and Ser\(^{378}\). In *in vitro* phosphorylation assays were performed using GST fusion proteins harboring serine-to-alanine mutations. Phosphorylation by CK-1 was reduced in GST-[S101A]parN (Fig. 6B) and GST-[S378A]parC (Fig. 6C). We detected slightly reduced phosphorylation of GST-[S101A]parN, GST-[S131A]parN and GST-[S136A]parN by PKA, which indicates that PKA might possibly phosphorylate these serine sites. On the other hand, PKC-mediated phosphate incorporation was not reduced in the serine-to-alanine mutants investigated (Fig. 6, B and C). Thus, PKC was not responsible for phosphorylation of these sites.

We further analyzed whether CK-1, PKA and PKC phosphorylate GST-parN and GST-parC in cell lysates. Fusion proteins were incubated with extracts prepared from HEK293 cells, \([\gamma-\text{P}]\text{ATP}\), and OA in the presence or absence of selective inhibitor of CK-1 (hymenialdisine), an inhibitor of PKA (H-89), and an inhibitor of PKC (GF 109203X). PDBu was used to stimulate PKC activity. Consistent with the result from *in vitro* phosphorylation assays (Fig. 6), we found that GST-parN and GST-parC were phosphorylated by cellular extracts in the absence of inhibitors (Fig. 7, A and B). Both parN and parC phosphorylation was completely inhibited with the CK-1 inhibitor hymenialdisine (Fig. 7, A and B), but not in the presence of the PKA inhibitor H-89. Stimulation of PKC with PDBu enhanced phosphorylation of parN and parC, and this effect was reversed with the PKC inhibitor GF 109203X.

Consistent with the *in vitro* phosphorylation assays (Fig. 6), each of the serine-to-alanine mutants (S101A, S131A, S136A, S296A, and S378A) showed reduced phosphorylation in cell lysates (Fig. 7, A and B). The incorporation of phosphate was completely abolished when GST-[S378A]parN was incubated with HEK293 lysates. Phosphate incorporation into the S296A mutant was also reduced in this assay, but to a lesser extent. Taken together, parkin is phosphorylated by CK-1, PKA, and
PKC. CK-1 is a strong candidate kinase for phosphorylation of Ser^{101} in the parkin N terminus and Ser^{378} in the parkin C terminus (Fig. 7C).

**Cellular Modulation of Parkin Phosphorylation**—After having identified experimental conditions affecting parkin phosphorylation, we studied how cellular stress would influence parkin phosphorylation. Parkin has been shown to reduce oxidative stress (35) and protect against unfolded protein stress mediated by proteasome inhibitors (19) and ER stress (8, 11, 18). Thus, we have exposed HEK293 cells stably transfected with MYC-parkin-V5 to hydrogen peroxide, the proteasome inhibitor MG132, and the glycosylation inhibitor tunicamycin, concomitant with stabilization of phosphorylation by OA. As an alternative means to induce ER stress, cells were transfected with PaelR-FLAG, an aggregation-prone ER protein that mediates ER stress upon overexpression (11). Indeed, aggregation of PaelR-FLAG was readily detected on FLAG-probed Western blots (Fig. 8A).

ER stress was confirmed by determination of the co-regulated ER chaperones Grp78 and Grp94 (36, 37). Tunicamycin treatment caused induction of GRP78 mRNA, as evidenced by RT-PCR, as well as Grp78 and Grp94 proteins, as evidenced by immunoblotting (Fig. 8B). PaelR overexpression caused an induction of these ER chaperones in HEK293 cells, an effect that was greatly reduced in parkin transfectants (Fig. 8B). Parkin expression also attenuated tunicamycin-induced glucose-regulated protein induction (Fig. 8B), consistent with previous reports that parkin protects cells against ER stress (8, 11).

[^32P]Orthophosphate incorporation into V5-immunoprecipitated MYC-parkin-V5 was found to be specifically reduced under conditions of protein folding stress. ER stress elicited by tunicamycin treatment and by PaelR overexpression greatly reduced overall parkin phosphorylation (Fig. 8). Proteasomal inhibition with MG-132 reduced parkin phosphorylation, but less than ER stress (Fig. 8). In contrast, oxidative stress mediated by hydrogen peroxide exposure did not affect parkin phosphorylation, although cytotoxicity became apparent after prolonged exposure to 60 μM H_{2}O_{2}. No ER stress was evidenced after H_{2}O_{2} treatment (Fig. 8B). Thus, protein folding stress specifically reduces parkin phosphorylation.

**OA-stabilized Phosphorylation of Parkin Suppresses E3 Activity**—In order to examine whether phosphorylation can affect the enzymatic activity of parkin, in vitro ubiquitination assays were performed. Since it has been reported that parkin is autoubiquitinated (7–9), we used parkin itself as a substrate. For this purpose, HEK293T cells were transfected with FLAG-parkin as well as phosphoserine mutants. After OA or control treatment, cell lysates were prepared, and lower panels reveal equal transfection efficiencies by immunoblotting (IB) using anti-V5 antibody. The closed arrows point to MYC-parN bands, and open arrows point to band-shifted, phosphorylated bands. B. MALDI-TOF mass spectrum of MYC-parN-V5 treated with OA after in-gel digest with endoproteinase Lys-C. The masses of the internal standards were as follows: MH^{+} = 2465.20-Da monoisotopic mass of ACTH-(18–39) and MH^{+} = 904.47-Da monoisotopic mass of des-Arg-bradykinin. Parkin-derived fragments were as follows: MH^{+} = 1488.76-Da monoisotopic mass of unphosphorylated Lys-C peptide Asp^{130} to Asn^{144}, MH^{+} = 1568.71-Da monoisotopic mass of phosphorylated Lys-C peptide Asp^{130} to Asn^{144}, and MH^{+} = 1755.03-Da monoisotopic mass of Lys-C peptide Arg^{23} to Lys^{48}. C, the tandem mass spectrum of the doubly charged precursor ion (m/z 784.8) allowed the identification of the phosphorylation site within the peptide DSPPAG-SPAGRSIYN obtained after in-gel digestion with endoproteinase Lys-C. The phosphorylated amino acid and product ions derived from y ions by \beta-elimination of phosphoric acid are assigned. D, experimental procedures were carried out as described in A, except that serines 296, 378, 384, and 407 in MYC-parC-V5 were individually mutagenized to alanine.
anti-FLAG immunoprecipitates were added for reconstitution of an in vitro ubiquitination assay. The formation of high molecular weight smears of biotin-ubiquitin in the FLAG immunoprecipitates revealed autoubiquitination of parkin. Overall phosphorylation upon OA treatment of wild-type parkin-transfected cells caused some reduction of parkin autoubiquitination (Fig. 9A).

To provide more quantitative measures of the effect of phosphorylation on parkin activity, we conducted autoubiquitination assays using recombinant GST-parkin phosphorylated in vitro with CK-1, PKA, and PKC. Parkin phosphorylation by these kinases reduced parkin activity (Fig. 9B).

In vitro phosphorylation of GST-parkin decreased its autoubiquitination activity by 24\% (n = 4) in the case of CK-1, by 44\% (n = 3) in the case of PKA, and by 39\% (n = 3) in the case of PKC (Fig. 9C). Thus, phosphorylation of parkin appears to down-regulate its ubiquitin ligase activity.

In the attempt to identify individual regulatory phosphorylation sites within parkin, we investigated FLAG-tagged constructs of the phosphorylation site serine-to-alanine and -aspartate mutants identified above. None of the individual phosphorylation sites investigated appeared to exert a unique regulatory role, as evidenced from densitometric quantification of the autoubiquitinated parkin bands (results not shown). Thus, if parkin E3 activity is regulated by phosphorylation, it must arise from multiple sites.

**DISCUSSION**

Here we demonstrate that parkin is phosphorylated both in nonneuronal and neuronal cell lines. Parkin appears to be dephosphorylated rapidly under steady state conditions, because the phosphate incorporation was hardly observed without stabilization with OA. At least 5 serine residues were identified as phosphorylation sites. The kinases CK-1, PKA, and PKC were found to phosphorylate parkin. In cells exposed to the Parkinson’s disease relevant protein folding stress (38), overall parkin phosphorylation decreased. Unphosphorylated parkin tended to be more active. These findings suggest that phosphorylation of parkin contributes to the regulation of its ubiquitin ligase activity upon unfolded protein stress.

Phosphoamino acid analysis revealed that serine sites are mainly phosphorylated in OA-treated cells. Threonine residues may also be phosphorylated in parkin, because a weak signal was clearly detected. Site-directed mutagenesis combined with in vivo and in vitro phosphorylation assays led to the identification of Ser101, Ser131, Ser136, Ser296, and Ser378 as phosphorylation sites in parkin. The corresponding serine-to-alanine mutants showed reduced, but not abolished incorporation of phosphate. Thus, multiple phosphorylation sites exist in parkin.

CK-1, PKA, and PKC were identified as putative parkin kinases. Specifically, CK-1 is one kinase to phosphorylate Ser101 and Ser278, because mutations of these sites to alanine strongly reduced incorporation of phosphate. However, the possibility cannot be excluded that other kinases are involved in phosphorylation at these serine sites. CK-1 is an unexpected kinase to phosphorylate parkin because there is no CK-1 recognition consensus sequence ((D/E)(X)XX(S/T)) in the amino acid sequence of parkin. CK-1 is ubiquitously expressed and involved in various important cellular processes, including signal transduction. We also observed a slightly reduced incorporation of phosphate by PKA in S101A, S131A, and S136A, which means that PKA may contribute to phosphorylation of these
Fig. 7. Phosphorylation of N terminus and C terminus by the extracts from HEK293 cells. A and B, cellular extracts from HEK293 cells were incubated with GST-parN (A), GST-parC (B), and various serine-to-alanine mutants plus [γ-32P]ATP and 4 μM OA in the presence or absence of CK-1-selective inhibitor hymenialdisine (5 μM), PKA-selective inhibitor H-89 (5 μM), and PKC-selective inhibitor GF 109203X (5 μM). In order to stimulate PKC, 1 μM PDBu was added. After the incubation, the precipitation using glutathione-Sepharose was carried out and subjected into 12% SDS-PAGE. Phosphorylated fusion proteins were detected by autoradiography (upper panels). Coomassie stain was carried out to prove the equal protein loading (lower panels). C, the arrows indicate phosphorylation sites in parkin. DMSO, Me₂SO.

Fig. 8. Phosphorylation of parkin is reduced by ER stress. A, HEK293 cells stably expressing MYC-parkin-V5 were subjected to in vivo phosphorylation assay directly (lanes 1–4) or 24 h after transient transfection with PaelR-FLAG (lane 5). During the 32PO₄ labeling, 1 μM OA (lanes 1–5) plus no additives (lanes 1 and 5), 60 μM H₂O₂ (lane 2), 20 μg/ml tunicamycin (lane 3), or 50 μM MG132 (lane 4) were administered. Anti-V5 immunoprecipitates were subjected to 12% SDS-PAGE. After electroblotting, phosphorylated proteins were detected by autoradiography (upper panel), and equal expression/loading was confirmed by probing with anti-V5 (middle panel). Overexpression and aggregation of PaelR-FLAG was confirmed on parallel immunoblots (IB) probed with anti-FLAG (lower panel). B, ER stress was determined by immunoblotting of Grp78 and Grp94 protein (upper panels) and RT-PCR of GRP78 mRNA (lower panels) in HEK293 cells stably transfected with MYC-parkin-V5 (lanes 1–5) or in untransfected HEK293 cells (lanes 6–10). All cells were treated for 3.5 h with 1 μM OA plus no additives (lanes 1, 5, 6, and 10), 60 μM H₂O₂ (lanes 2 and 7), 20 μg/ml tunicamycin (lanes 3 and 8), or 50 μM MG132 (lanes 4 and 9).
Parkin Phosphorylation and Modulation of Its E3 Activity

Fig. 9. Phosphorylation down-regulates parkin autoubiquitination activity. A, cellular extracts from transfected HEK293T cells in the presence or absence of OA and Me₂SO were subjected to immunoprecipitation (IP) using FLAG M2-agarose. Immunoprecipitates were incubated with E1, E2 (UbcH7), and biotin-ubiquitin at 30 °C for 90 min reaction (rxn) time (upper panel). Reaction mixtures were subjected to 10% SDS-PAGE and transferred onto PVDF membrane. Ubiquitinated parkin proteins were analyzed by immunoblot (IB) with streptavidin-binding biotinylated ubiquitin (upper panel). Total lysates were analyzed by immunoblot with anti-FLAG antibody (lower panel). B, GST-parkin immobilized on glutathione-Sepharose was phosphorylated by CK-1, PKA, or PKC. The same experimental procedure was carried out as described in A. C, signal intensities of the streptavidin-binding biotinylated ubiquitin smears generated in experiments performed as described in B were quantified by densitometric scanning and expressed as a percentage of unphosphorylated parkin activity. Error bars, S.E. of four (CK-1) and three (PKA and PKC) experiments, respectively.

sites. PKC is able to phosphorylate parkin but is not responsible for the serine sites investigated here. Since three identified phosphorylation sites were located in the linker region, and it has been reported that the ubiquitin-like domain interacts with Rpn10 in proteasome (39), we also investigated whether parkin phosphorylation could affect proteasome activities. Each serine-to-alanine or -aspartate mutants still had almost the same level of activities as vector controls (data not shown). The RING-IBR-RING motif of parkin is important to interact with E2 co-enzymes. However, S296A and S378A did not consistently show reduced levels of ubiquitination by in vitro ubiquitination assays (data not shown). Although the RING-IBR-RING motif is crucial for parkin ubiquitin ligase function, single site phosphorylation in this domain appears to have no effect on autoubiquitination. Nevertheless, OA-mediated overall phosphorylation of parkin slightly reduced its E3 enzymatic activity. The regulation of parkin E3 activity must be due to multiple phosphorylation sites.

ER stress (but not oxidative stress) was found to specifically reduce parkin phosphorylation levels. Specifically, we found that OA-stabilized phosphorylation of parkin or phosphorylation by identified parkin kinases caused a small but significant reduction of parkin autoubiquitination activity. More in vitro work is needed to elucidate if and how parkin phosphorylation affects the activity and recognition of the various substrates of the E3 ubiquitin ligase parkin. In fact, some of the polyubiquitin signals detected in the in vitro E3 assay (Fig. 9) may arise from ubiquitination of co-purified, parkin-associated ubiquitin ligase substrates.

Because CK-1 appeared to be a major parkin kinase (Fig. 7), further investigations of signal transduction events involving CK-1 might be particularly revealing for the regulation of parkin E3 ubiquitin ligase activity. More generally, the involvement of parkin phosphorylation in the ER unfolded protein stress response (Fig. 8) might contribute to the understanding of parkin as dopaminergic neuron survival factor.

Taken together, we suggest that the reduced phosphorylation of parkin in ER stressed cells contributes to the up-regulation of parkin E3 ubiquitin ligase activity, which is believed to suppress cytotoxicity due to unfolded protein stress.

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Ayako Yamamoto, Arno Friedlein, Yuzuru Imai, Ryosuke Takahashi, Philipp J. Kahle and Christian Haass

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