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Mitochondrial hexokinase HKI is a novel substrate of the Parkin ubiquitin ligase

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ABSTRACT

Dysfunction of Parkin, a RING-IBR-RING motif containing protein, causes autosomal recessive familial Parkinsonism. Biochemically, Parkin is a ubiquitin-ligating enzyme (E3) that catalyzes ubiquitin transfer from ubiquitin-activating and -conjugating enzymes (E1/E2) to a substrate. Recent studies have revealed that Parkin localizes in the cytoplasm and its E3 activity is repressed under steady-state conditions. In contrast, Parkin moves to mitochondria with low membrane potential, thereby activating the latent enzymatic activity of the protein, which in turn triggers Parkin-mediated ubiquitylation of numerous mitochondrial substrates. However, the mechanism of how Parkin-catalyzed ubiquitylation maintains mitochondrial integrity has yet to be determined. To begin to address this, we screened for novel Parkin substrate(s) and identified mitochondrial hexokinase I (HKI) as a candidate. Following a decrease in membrane potential, Parkin ubiquitylation of HKI leads to its proteasomal degradation. Moreover, most disease-relevant mutations of Parkin hinder this event and endogenous HKI is ubiquitylated upon dissipation of mitochondrial membrane potential in genuine-Parkin expressing cells, suggesting its physiological importance.

1. Introduction

Parkinson's disease (PD) is one of the most pervasive neurodegenerative diseases affecting 1% of the population over the age of 65. PD manifests as the dysfunction and loss of dopaminergic neurons in the *substantia nigra,* although neurons in other brain regions are also affected. PD commonly arises sporadically; however, in some cases the disease is familial and inherited. *PARKIN* is a causal gene for autosomal recessive early-onset parkinsonism [1] the product of which (the Parkin protein) functions as an enzyme responsible for substrate recognition and subsequent ubiquitin-ligation (i.e. E3 enzyme) [2].

Mitochondrial homeostasis plays a pivotal role in the maintenance of normal healthy cells, in particular non-dividing cells such as neurons. To maintain the integrity of mitochondria, the selective elimination of impaired mitochondria caused by various endogenous and exogenous stresses, such as unnecessary generation of reactive oxygen species (ROS) and mtDNA mutations, is critical [3,4]. Newly emergent evidence has shown that Parkin-dependent ubiquitylation plays a pivotal role in the quality control of mitochondria. The linkage between Parkin and mitochondria was first discovered in loss-of-function mutations in the fruit fly [5]. *Drosophila* that lack Parkin display severe defects in mitochondria [5]. Later, a reduction in the respiratory capacity of striatal mitochondria was demonstrated in parkin−/− mice [6]. In 2008, Richard Youle's lab reported that Parkin translocates to depolarized mitochondria and induces autophagic degradation of damaged mitochondria, a process termed mitophagy [7]. In 2010, several groups, including ours, reported that the recruitment of Parkin to impaired mitochondria requires PINK1, another early-onset hereditary PD gene product [8–11]. Following translocation to the mitochondrial surface, Parkin is converted to its active form [9] and ubiquitylates numerous substrates on the outer mitochondrial membrane (OMM) upon dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$). Parkin has been shown to ubiquitylate a translocase component of the OMM complex (Tom70, Tom40 and Tom20), the pro-apoptotic factors BAK and BAX, mitochondrial Rho GTPases (MIRO) 1 and 2, mitochondrial fission factors FIS1 and Drp1, mitochondrial fusion factor Mitofusin (MFN), and voltage-dependent anion channel VDAC [8,12–22]. Additionally, high-throughput analysis revealed that many other proteins are downregulated upon Parkin expression in HeLa cells [23], implying that more substrates of Parkin exist. Collectively, Parkin seems to

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ubiquitylate diverse substrates, thus regulating multiple aspects of mitochondrial biology.

To further explore the molecular function of Parkin, we screened for cellular partner(s) that interact with Parkin following mitochondrial depolarization, and identified a novel substrate, hexokinase I (HKI), that had eluded previous identification in high-throughput analyses [23]. HKI is ubiquitylated by Parkin upon dissipation of mitochondrial membrane potential. Here, we present data suggesting that HKI is a novel Parkin substrate.

2. Materials and methods

2.1. Plasmids and reagents

Plasmids to express wild type Parkin and various pathogenic mutants have been described previously [9,24,25]. HKI and CISD1/mitoNEET were amplified by PCR from a brain cDNA library and were sub-cloned into pcDNA3.1-HA vector. Plasmid transfection was performed using FuGene6 (Roche). To depolarize the mitochondria, cells were treated with 10–15 μM CCCP (Sigma) for 1 h, unless otherwise specified. To inhibit proteolytic activity of the proteasome or the lysosome, 10 μM MG132 (Peptide Inc.) or 200 nM Bafilomycin A1 (Wako) was added simultaneously with CCCP for the indicated time. To inhibit protein translation, 50 μg/ml cycloheximide (Wako) was used. To allow for PINK1 accumulation, cells were treated with cycloheximide 30 min after CCCP treatment.

2.2. Protein identification by LC-MS/MS analysis

Flag-Parkin was expressed in HEK293 cells and immunoprecipitated using an anti-Flag antibody (M2). Parkin-associated proteins were digested with lysyl endopeptidase C (Lys-C, Wako) and the resulting peptides mixtures diluted 10-fold with 0.1% formic acid were analyzed by direct nanoflow liquid chromatography tandem mass spectrometry (DNLC-MS/MS) system [26] coupled to a QSTAR XL (AB Sciex). Peptides were separated on a C18 reversed-phase column packed with Mightysil C18 (particle size 3 μm; Kanto Chemical) by a 40-min linear gradient from 5% to 40% acetonitrile in 0.1% formic acid, and were sprayed on-line to the mass spectrometer. All MS/MS spectra were queried against the National Center for Biotechnology Information (NCBI) non-redundant database using an in-house Mascot server (Matrix Science).

2.3. Immunofluorescence (IF), immunoprecipitation (IP) and immunoblotting (IB)

To detect the ubiquitylation of VDAC1, HKI, CISD1/mitoNEET and Tom70 in immunoblots, cell lysates were collected in the presence of 10 mM *N-ethylmaleimide* to protect ubiquitylation from deubiquitylating enzymes. For IF experiments, cells were fixed with 4% paraformaldehyde, permeabilized with 50 μg/ml digitonin, and stained with primary antibodies described below and the following secondary antibodies: mouse and/or rabbit Alexa Fluor 488, 568, and 647 (Invitrogen). Cells were imaged using a laser-scanning microscope (LSM510; Carl Zeiss, Inc.). Image contrast and brightness were adjusted in Photoshop (Adobe). Antibodies used are as follows: anti-VDAC1 (Ab-3; Calbiochem), anti-actin (AC-40; Sigma), anti-hemagglutinin (HA) (6E2; Cell Signaling), anti-Parkin (PRK8; Sigma) and anti-ubiquitin (P4D1; Santa Cruz Biotech.) For IB, the following were used: anti-Flag (M2; Sigma), anti-HKI (C35C4; Cell Signaling), anti-HA (F7; Santa Cruz), and anti-Tom20 (FL-145 and F-10; Santa Cruz Biotech.). An anti-Tom70 antibody for IB [27] was provided by Drs. T. Otera, T. Oka, and K. Mihara (Kyushu University).

3. Results

3.1. Seven proteins were identified as Parkin-binding molecules following CCCP treatment

To identify novel Parkin substrate(s), we screened for proteins that interact with Parkinin response to a decrease in the mitochondrial membrane potential. N-terminal Flag-tagged Parkin was expressed in HEK293 cells, which were subsequently treated with the mitochondrial uncoupler carbonyl cyanide *m-chlorophenylhydrazone* (CCCP) and immunoprecipitated with an anti-Flag antibody. The immunoprecipitates were eluted with a Flag peptide and digested with Lys-C endopeptidase. The cleaved fragments were directly analyzed using a nano-flow LC-MS/MS system. Following a database search, a dozen peptides were assigned to MS/MS spectra obtained from four nano-LC-MS/MS analyses of the Flag-Parkin associated proteins. Finally, seven proteins were identified: three variants of a voltage-dependent anion channel 1 (VDAC1, VDAC2, and VDAC3), Tom70, a CDGSH iron sulfur domain-containing protein 1 (CISD1)/mitoNEET, non-metastatic cells 2 (NME2)/NM23B, and hexokinase I (HKI) (Table 1). Among those proteins, NME2/NM23B has been categorized in the UniProt database (http://www.uniprot.org/) as a cytoplasmic protein. The other six identified proteins have been categorized by both UniProt and MitoCarta databases [28] as predominantly mitochondrial. Given that Parkin localizes to mitochondria following CCCP treatment [7], we focused on these six mitochondrial proteins in the ensuing experiments.

3.2. VDAC1, CISD1 and HKI co-expressed with Parkin undergo modification following CCCP treatment

We next examined whether the aforementioned proteins undergo Parkin-mediated ubiquitylation following a decrease in the mitochondrial membrane potential. BecauseVDAC2 and VDAC3 show high sequence similarity (91% amino acid identity) to VDAC1, VDAC1 was used as the representative of these VDAC family proteins. HeLa cells lack a functional *PARKIN* gene [29] and thus exogenous HA-Parkin was introduced into HeLa cells. Interestingly, a higher molecular mass population of endogenous VDAC1 was observed following CCCP treatment in HA-Parkin expressing cells (Fig. 1A). The modification resulted in 6–7 kDa increase in the molecular weight of VDAC with the pattern strongly suggestive of ubiquitylation by Parkin, as has been reported previously [8]. There are currently no reliable antibodies capable of detecting endogenous CISD1/mitoNEET, we consequently fused a hemagglutinin (HA)-tag to the C-terminus of CISD1/mitoNEET to facilitate detection (note that the N-terminal portion of CISD1/mitoNEET is the domain for the mitochondrial outer-membrane anchor [30]) and was thus not selected for the epitope tag]. When CISD1/mitoNEET-HA and Parkin were co-expressed in HeLa cells, a higher molecular mass population of CISD1/mitoNEET-HA (putative ubiquitylated form) was also observed following CCCP treatment (Fig. 1B). In exogenous Parkin-expressing HeLa cells, a similar shift in the molecular weight of endogenous Tom70 was detected following CCCP treatment (Fig. 1C); however, that signal was much fainter than that of VDAC1 or CISD1/mitoNEET (compare Fig. 1C with 1A/1B). Finally, we examined HKI, an enzyme that phosphorylates six-carbon sugars and forms hexose-phosphates such as glucose-6-phosphate. Because the N-terminal region of HKI functions as the mitochondrial localization signal [31], a HA tag was fused to the C-terminus for subsequent detection and immunoprecipitation. When HKI-HA and Parkin wereco-expressed in HeLa cells, a higher molecular mass population of HKI was clearly observed following CCCP treatment. This modification was completely depen-

Table 1
Isolated proteins that potentially interact with Parkin.

Swiss-Prot accession number	Gene	Protein name	Subcellular localization	Previous report of physical and/or functional interaction
P2179	VDAC1	Voltage-dependent anion-selective channel protein 1	Mitochondria	Ref. [8,23]
P4588	VDAC2	Voltage-dependent anion-selective channel protein 2	Mitochondria	None
Q9Y277	VDAC3	Voltage-dependent anion-selective channel protein 3	Mitochondria	None
P19367	HKI	Hexokinase-1	Mitochondria	None
O94826	TOMM70A	Mitochondrial import receptor subunit Tom70	Mitochondria	Ref. [23]
Q9NZ45	CISD1	CDGSH iron-sulfur domain-containing protein 1 (MitoNEET)	Mitochondria	Ref. [23]
P22392	NME2/ NM23B	Nucleoside diphosphate kinase B	Cytoplasm	Ref. [23]

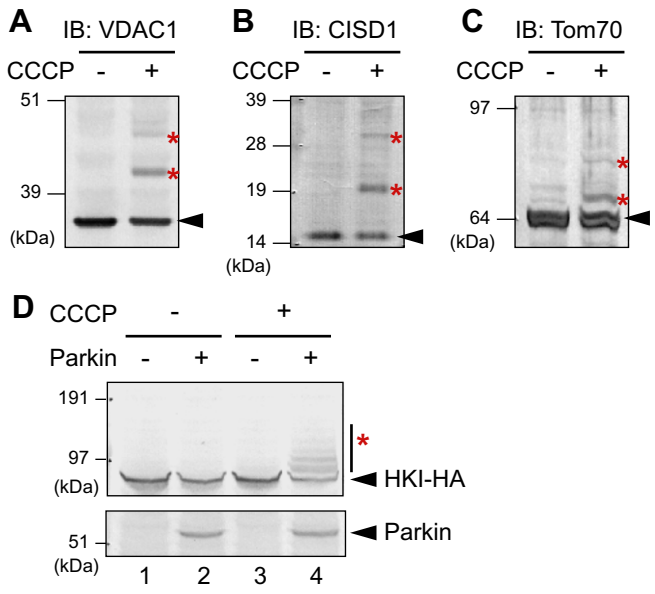

Fig. 1. (A–C) Isolated Parkin-interacting proteins underwent modification following a decrease in mitochondrial membrane potential. Endogenous VDAC1 (A), exogenous CISD1 (B) and endogenous Tom70 (C) were immunoblotted following CCCP treatment. (D) HeLa cells expressing both HKI and Parkin were treated with CCCP and then immunoblotted with the indicated antibodies. HKI underwent a ladder-like modification following Parkin and CCCP. Asterisks show Parkin-dependent modification.

dent on exogenous Parkin expression (Fig. 1D). In summary, VDAC1, CISD1/mitoNEET, and HKI were clearly modified, putatively via ubiquitylation, following a decrease in mitochondrial membrane potential whereas the signal for the modified Tom70 was much more faint. Ubiquitylation of VDAC1 and CISD1/mitoNEET following CCCP treatment have previously been reported [8,23], whereas ubiquitylation of HKI on depolarized mitochondria has not been reported. We thus selected HKI for further analyses.

3.3. Parkin-catalyzed ubiquitylation of hexokinase is blocked by pathogenic Parkin mutations

HKI-HA was immunoprecipitated with an anti-HA antibody and detected by anti-ubiquitin antibodies (Fig. 2A), demonstrating that the modification on HKI-HA was indeed ubiquitylation. We next examined the co-localization of Parkin and HKI in HeLa cells. Cells co-transfected with GFP-Parkin and HKI-HA were subjected to immunocytochemistry in the presence or absence of CCCP. An exogenous HKI-derived signal merged with a Tom20 signal irrespective of CCCP treatment, indicating the continuous mitochondrial localization of exogenous HKI, as reported previously [31]. GFP-Parkin localized throughout the cytoplasm under steady state conditions, but was transported to the mitochondria where it co-localized with HKI following CCCP treatment (Fig. 2B).

We next examined whether pathogenic mutations of Parkin affected the ubiquitylation of HKI. HA-Parkin mutants harboring one of eight pathogenic mutations (R42P, K161N, K211N, T240R, R275W, C352G, T415N and G430D) were serially introduced into HeLa cells with HKI-HA, followed by CCCP treatment for one hour, and the ubiquitylation of HKI analyzed. The R42P and R275W mutations destabilize exogenous Parkin, thus making interpretation of the results difficult because the diminution in HKI ubiquitylation is attributable to a decrease in mutant Parkin expression. Even though the expression of other pathogenic Parkin mutants was equivalent to wild type Parkin, HKI ubiquitylation in those cells was severely compromised (Fig. 2C). These results demonstrate that most of the pathogenic mutations of Parkin inhibit the ubiquitylation of HKI.

3.4. HKI is degraded by the proteasome following CCCP treatment

We next determined if HKI is destined for proteasomal degradation following Parkin-dependent ubiquitylation. HKI-expressing cells were treated with CCCP for 30 min (during this time PINK1 accumulates on mitochondria) and then subjected to CCCP and cycloheximide (CHX), an inhibitor of protein biosynthesis, simultaneously. In the presence of CHX, HKI underwent ubiquitylation one hour following CCCP treatment (Fig. 3A, lane 2) but gradually decreased over six hours (Fig. 3A, lanes 3,4). We thus examined whether the proteasomal inhibitor MG132 blocked the degradation of ubiquitylated HKI at six hours. When cells were treated with MG132, ubiquitylated HKI accumulated (Fig. 3B, compare lane 4 with 5) whereas treatment with bafilomycinA_1, an inhibitor of vacuolar-type H+-ATPase that prevents intra-lysosomal degradation, had no effect on the accumulation of ubiquitylated HKI (Fig. 3B, lane 6). These results suggest that Parkin-dependent ubiquitylation of HKI following dissipation of the mitochondrial membrane potential leads to its proteasomal degradation.

3.5. Endogenous HKI is ubiquitylated following CCCP treatment in cells expressing genuine Parkin

Although a clear ubiquitylation signal on HKI was observed (Fig. 2), the experimental conditions were artificial because both HKI and Parkin was over-produced to demonstrate the ubiquitylation. We thus used more stringent criteria to examine whether endogenous HKI is ubiquitylated following a decrease in the mito-

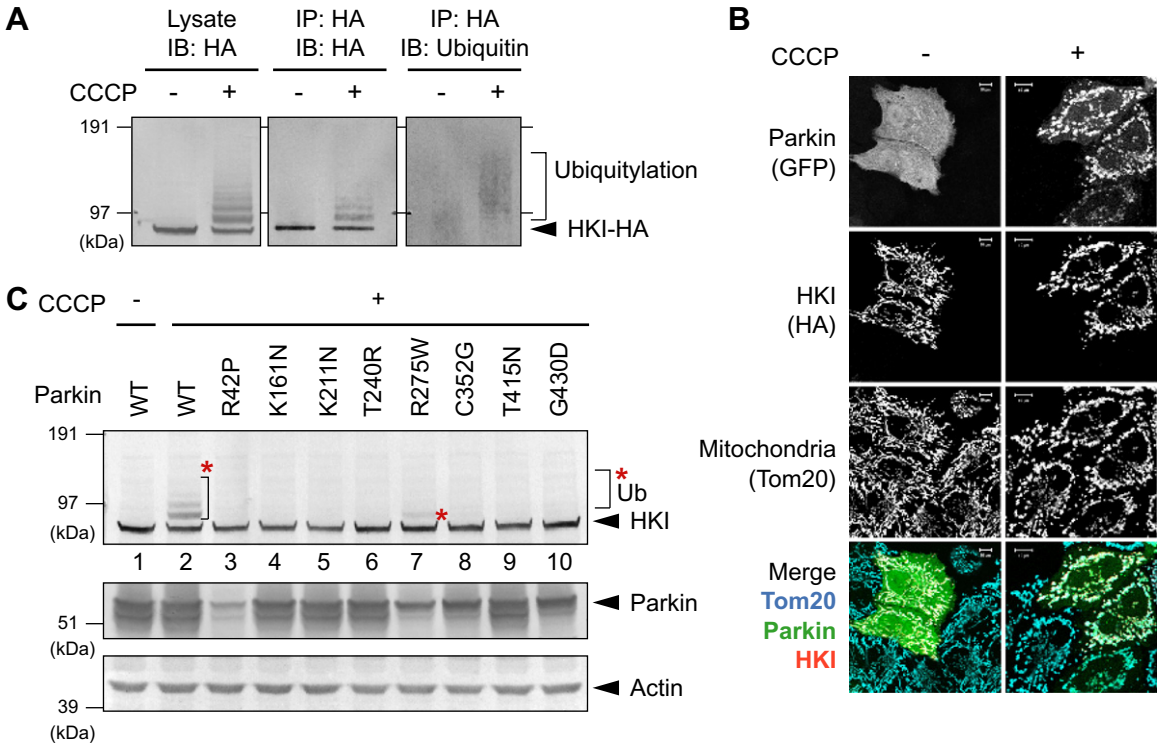

Fig. 2. HKI ubiquitylation on damaged mitochondria is inhibited by pathogenic mutations of Parkin. (A) HeLa cells expressing HKI-HA were treated with CCCP, immunoprecipitated with an anti-HA antibody and immunoblotted with anti-ubiquitin antibody. The result reveals that HKI is ubiquitylated. (B) HeLa cells expressing both GFP-Parkin and HKI-HA +/− CCCP treatment were immunostained. The results show that Parkin co-localized with HKI on damaged mitochondria. Bars, 10 μm. (C) HeLa cells expressing HKI and Parkin mutants were treated with CCCP and immunoblotted. HKI ubiquitylation was severely compromised by various pathogenic mutations in Parkin.

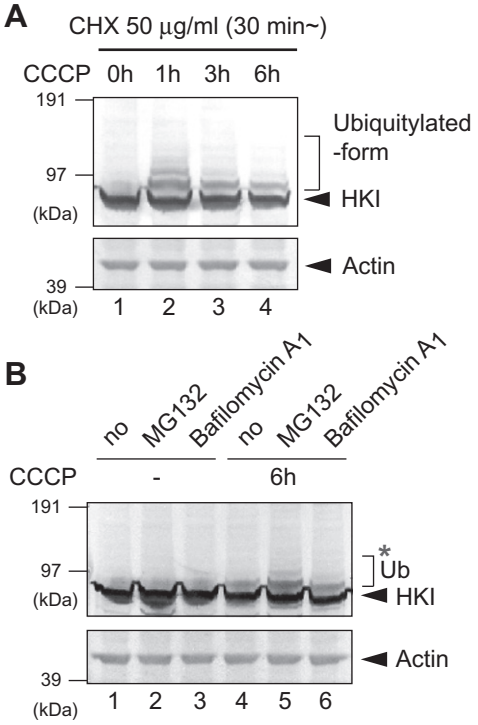

Fig. 3. (A) Degradation of HKI by the proteasome. HeLa cells were treated with CCCP and cycloheximide, and immunoblotted with the indicated antibodies. (B) Ubiquitylated HKI accumulated following treatment with MG132, a proteasome inhibitor. HeLa cells treated with CCCP, cyclohexmide and MG132 or Bafilomycin A1 were immunoblotted with the indicated antibodies.

chondrial membrane potential in cells expressing endogenous Parkin. As reported previously, HEK293 cells possess endogenous Parkin [32] and they also express endogenous HKI (Fig. 4A). When HEK293 cells were subjected to immunocytochemistry, endogenous HKI localized on mitochondria both in the presence and absence of CCCP (Fig. 4B), consistent with that of exogenous HKI (Fig. 2B). CCCP treatment of HEK293 cells resulted in a faint modification of HKI (Fig. 4C, a red asterisk). Clearer results were obtained following immunoprecipitation with an anti-HKI antibody. HKI was observed as doublet (Fig. 4C, a blue asterisk) and the upper band was detected with an anti-ubiquitin antibody (Fig. 4C, a black asterisk). We thus concluded that endogenous HKI also undergoes ubiquitylation in response to dissipation of the mitochondrial membrane potential in endogenous-Parkin-expressing cells.

4. Discussion

Our understanding of Parkin function has greatly expanded in recent years and shown to work with PINK1 to identify, label, clear and quarantine damaged mitochondria in cells (reviewed in [3,4,33]). Biochemically, Parkin functions as an E3 enzyme that catalyzes ubiquitin transfer from E1/E2~ubiquitin to the substrate [2,34–38]. Because PD is one of the most prevalent neurodegenerative disorders, the identification of Parkin substrates is important not only scientifically but also in terms of public welfare. Consequently, a number of Parkin substrates (>25) have been identified. However, there is one provision to most of these experiments, i.e. over-expressed Parkin and/or over-produced substrates were used to show the ubiquitylation. Studies without overexpression of the exogenous gene are extremely limited, excluding a few exceptions such as MFN [18].

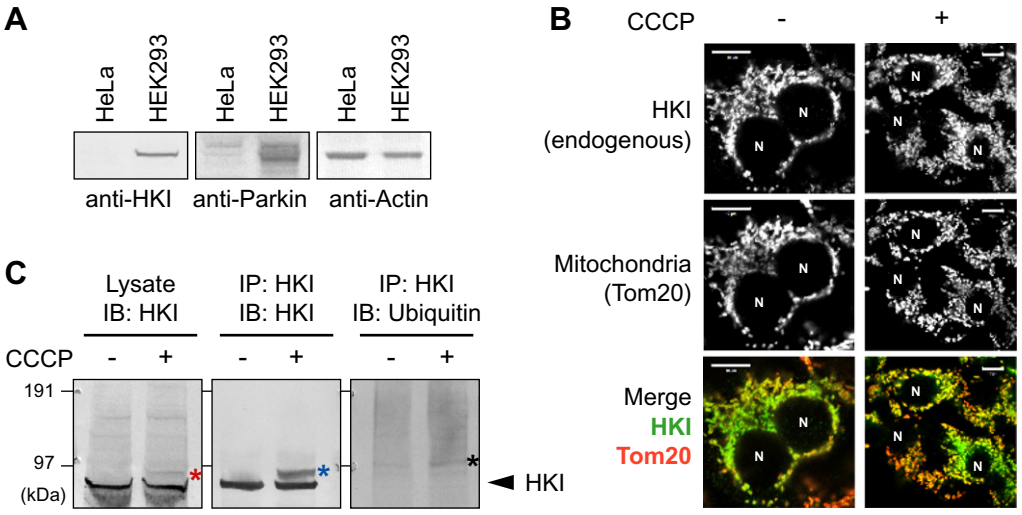

Fig. 4. (A) Expression of endogenous Parkin and HKI in HeLa and HEK293 cells. Actin was used as a loading control. (B) Immunocytochemistry of native HEK293 cells shows endogenous HKI localized on mitochondria regardless of CCCP treatment. N, nuclei; Bars, 10 μm. (C) Endogenous HKI was ubiquitylated following CCCP treatment in HEK293 cells. Lysates of untransfected HEK293 cells following CCCP treatment were immunoprecipitated with an anti-HKI antibody and immunoblotted with an anti-ubiquitin antibody.

To further reveal the function of Parkin, we sought to identify additional substrate(s) that are ubiquitylated following a decrease in mitochondrial membrane potential. In this study, we identified hexokinase I (HKI) as a candidate substrate. Exogenous HKI was ubiquitylated by exogenous Parkin (Fig. 1D). Moreover, we showed that endogenous HKI is also ubiquitylated upon dissipation of the mitochondrial membrane potential in Parkin-possessing cells (Fig. 4), whereas in cells lacking endogenous Parkin (e.g. HeLa cells) HKI is not ubiquitylated following CCCP treatment and exogenous-Parkin-expression is required for the ubiquitylation (Fig. 1). Attempts to use siRNA knockdown of endogenous Parkin in HEK cells were problematic as only a subtle decrease in the ubiquitylation of positive controls Mitofusin and Miro was observed (data not shown). We thus have not unequivocally demonstrated that endogenous HKI is ubiquitylated by endogenous Parkin following a decrease in the mitochondrial membrane potential in cells and further analysis will be required. Nevertheless, our results strongly suggest that HKI is a genuine novel substrate of Parkin.

Hexokinase plays a pivotal role in the cellular uptake and utilization of glucose. Hexokinase catalyzes the first obligatory step of glucose metabolism, namely the ATP-dependent phosphorylation of glucose to yield glucose-6-phosphate. Mammalian cells possess four highly homologous hexokinase isoforms, i.e. HKI, HKII, HKIII, and HKIV/glucokinase. HKI is predominantly expressed in the brain and the kidney, whereas HKII is expressed in muscle and adipose tissue. An important distinguishing feature of the HKI and HKII isoforms is mitochondrial localization via direct interaction with VDAC, the latter constituting a mitochondrial permeability transition pore (MPTP) [31,39]. We speculate that Parkin-catalyzed ubiquitylation of HKI perturbs MPTP function on depolarized mitochondria. In another scenario, hexokinase may link Parkin to the intracellular metabolic pathway, i.e. Parkin negatively regulates the pentose phosphate pathway and glycolysis via degradation of HKI. Interestingly, Van Laar et al. [40] reported that Parkin translocates to damaged mitochondria under glycolytic metabolism, whereas Parkin does not localize to depolarized mitochondria in cells forced into dependence on mitochondrial respiration. If so, then Parkin-mediated HKI degradation may inhibit glycolysis, and consequently Parkin's mitochondriallocalization is hindered, suggesting HKI functions as a negative feedback component.

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