Published in final edited form as:

J Am Soc Mass Spectrom. 2020 May 06; 31(5): 1132–1139. doi:10.1021/jasms.0c00058.

Quantitative Middle-Down MS Analysis of Parkin-Mediated Ubiquitin Chain Assembly

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Abstract

Misregulation of the E3 ubiquitin ligase Parkin and the kinase PINK1 underlie both inherited and idiopathic Parkinson's disease-associated neurodegeneration. Parkin and PINK1 work together to catalyze the assembly of ubiquitin chains on substrates located on the outer mitochondrial membrane to facilitate autophagic removal of damaged mitochondria through a process termed mitophagy. Quantitative measurements of Parkin-mediated chain assembly, both *in vitro* and on mitochondria, have revealed that chains are composed of Lys6, Lys11, Lys48, and Lys63 linkages. The combinatorial nature of these chains is further expanded by the ability of PINK1 to phosphorylate individual subunits. The precise architecture of chains produced by the coordinated action of PINK1 and Parkin, however, are unknown. Here, we demonstrate that quantitative middle-down mass spectrometry using uniformly ¹⁵N-labeled ubiquitin variants as internal standards informs on the extent of chain branching. We find that Parkin is a prolific branching enzyme *in vitro*. Quantitative middle-down mass spectrometry also reveals that phospho-Ser65-ubiquitin (pSer65-Ub)—a key activator of Parkin—is not incorporated into chains to a significant extent. Our results suggest that Parkin-mediated chain branching is “on-pathway”, and branch points are the principal targets of the deubiquitinase USP30.

Graphical Abstract

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Complete contact information is available at: <https://pubs.acs.org/10.1021/jasms.0c00058>

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jasms.0c00058>.

Figures S1–S4 (PDF)

Tables S1–S3 (XLSX)

The authors declare no competing financial interest.

Keywords

post-translational modifications; ubiquitin; ubiquitin chains; polyubiquitin; phospho-ubiquitin; E3 ligase; Parkin; mitophagy; middle-down mass spectrometry

INTRODUCTION

Parkinson's disease (PD) is a devastating neurological disorder characterized by the loss of dopaminergic neurons that affects 7–10 million people worldwide.¹ Although the etiology is largely unknown, defects in oxidative phosphorylation are thought to contribute to PD pathogenesis.^{2–4} When errors occur, reactive oxygen species accumulate, resulting in damaged mitochondria, which must be rapidly disposed of through a process called mitophagy to avoid detrimental effects on cellular physiology. The E3 ubiquitin (Ub) ligase Parkin and the kinase PINK1 play key roles in mitophagy.^{5–7} Somatic mutations in the genes encoding Parkin (*PARK2*) and PINK1 (*PARK6*) are directly linked to an early onset form of the disease known as autosomal-recessive juvenile Parkinsonism (AR-JP), which accounts for 5–10% of all cases.^{8,9}

Over the last 5 years, the mechanism by which Parkin and PINK1 promote mitophagy has been elucidated.^{10–17} Following damage, PINK1 accumulates on the mitochondrial outer membrane (MOM), where it activates Parkin *via* a multistep feedforward mechanism. During the initial stages, PINK1 phosphorylates Ser65 of Ub (pSer65-Ub) to recruit auto-inhibited cytosolic Parkin.^{18–20} The pSer65-Ub–Parkin interaction stimulates Ub ligase activity, resulting in the formation of ubiquitin chains, which also serve as substrates of PINK1 to further retain and activate Parkin. Another consequence of the pSer65-Ub–Parkin interaction is that it releases the N-terminal Ub-like (Ubl) domain from the core of Parkin, increasing the rate at which PINK1 phosphorylates Ser65 of the Ubl domain.^{14,21–26} Once Parkin is phosphorylated, it is stabilized in an open and active conformation, and the binding affinity to pSer65-Ub is enhanced by ~20-fold.²⁷ Thus, the pSer65-Ub–pSer65-Parkin complex supports full retention and activation to ultimately afford a high density of Ub chains to mark damaged mitochondria for mitophagy. The precise architecture of these chains is unknown. Considering the MOM-bound chains are decoded by a set of Ub chain-binding autophagy receptors that control the flux through mitophagy, it is imperative to gain deeper insight into the architecture of chains built by Parkin.^{28–30}

There is an astounding number of possible Ub chains that can be synthesized on the MOM. Ub has seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) and an amino terminus (Met1), allowing for chains to be assembled using a single linkage (homotypic) or a mixture of linkages (heterotypic). Branched chains can also emerge when a Ub subunit is modified at more than one amino group. *In vitro*, Parkin catalyzes the formation of chains bearing Lys6, Lys11, Lys48, and Lys63 linkages.^{15,27,31,32} The same linkages are generated in mitochondrial depolarized cells overexpressing Parkin, but in depolarized embryonic stem-cell-derived neurons, the prevailing linkage type is Lys63.³³ On top of this complexity, PINK1 can phosphorylate any chain type and, in principle, any subunit, although there is evidence that phosphorylation at the distal position is preferred.³⁴ The combinatorial nature of these modifications presents a significant analytical challenge for defining chain architecture. Most methods rely on information from tryptic peptides to report on linkage composition and the extent of phosphorylation. However, information on the architecture of chains is lost.

Our lab has previously shown that the combination of limited trypsinolysis and intact MS enables the characterization of multiple modifications on a single Ub subunit and can inform on chain architecture.^{35–37} In the context of Parkin-derived chains, the challenge is that phosphate groups are known to affect ionization efficiency, making it difficult to quantify the abundance of Ub and pSer65-Ub in their unmodified and modified forms. Here, we sought to address this problem by developing a method to quantify Ub and pSer65-Ub derivatives in chain assembly reactions with Parkin alone and in the presence of the mitochondrial deubiquitinase USP30.^{32,34,38}

MATERIALS AND METHODS

Protein Expression and Purification.

E1 was purified as previously described.³⁹ Briefly, N-terminally His6-tagged E1 was expressed in Rosetta 2(DE3) pLysS *Escherichia coli* cells in LB media supplemented with appropriate antibiotics at 37 °C to OD₆₀₀ ~ 0.6–0.8 and transferred at 18 °C for 16 h after induction with 250 μM IPTG. Cultures were harvested, flash frozen, resuspended in lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA and 10 mM imidazole), lysed by sonication, and clarified at 60,000g for 30 min at 4 °C. Clarified lysate was then incubated with Ni-NTA resin for 1 h, washed with lysis buffer, and eluted into Ni-NTA elution buffer (lysis buffer plus 300 mM imidazole).

UBE2L3 was purified as previously described.³⁵ N-Terminally GST-tagged UBE2L3 was expressed in Rosetta 2(DE3) pLysS *E. coli* cells in LB media supplemented with appropriate antibiotics at 37 °C to OD₆₀₀ ~ 0.6 and transferred to 16 °C for 16 h after induction with 250 μM IPTG. Cultures were harvested, flash frozen, resuspended in lysis buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT), lysed by sonication, and clarified at 30,000g for 30 min at 4 °C. Clarified lysate was then incubated with GST resin for 1 h, washed with lysis buffer, resuspended into 3C protease buffer (50 mM Tris pH 7.5 and 150 mM NaCl), and cleaved overnight with 3C protease.

N-Terminally His6-SUMO-tagged Parkin was expressed in Rosetta 2(DE3) pLysS *E. coli* cells in LB media supplemented with appropriate antibiotics and 300 μ M ZnCl₂ at 37 °C to OD₆₀₀ ~ 0.6 and transferred to 16 °C for 12 h after induction with 50 μ M IPTG. Cultures were harvested, flash frozen, resuspended in lysis buffer (75 mM Tris HCl pH 7.4, 0.5 mM TCEP, 500 mM NaCl, 1 mM benzamide, 0.1 mM PMSF in the presence of DNase I), lysed by sonication, and clarified at 30,000g for 30 min at 4 °C. Clarified lysate was then incubated with Ni-NTA resin for 1 h, washed with 75 mM Tris HCl pH 7.4, 0.5 mM TCEP, 500 mM NaCl, and eluted from the resin using an imidazole gradient (wash buffer containing 100 mM imidazole and then again with 300 mM imidazole). Parkin was further purified using gel filtration (Superdex 75, GE Life Sciences) with 50 mM Tris HCl pH 7.4 and 300 mM NaCl.

N-Terminally His6-MBP-tagged *Tribolium castaneum* PINK1 (*TcPINK1*) was expressed in BL21 (DE3) pLysS *E. coli* cells in LB media supplemented with appropriate antibiotics at 37 °C to OD₆₀₀ ~ 0.6 and transferred to 16 °C for 16 h after induction with 250 μ M IPTG. Cultures were harvested, flash frozen, resuspended in lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5% glycerol, 1% Triton X-100, 0.1 mM TCEP, 1 mM benzamidine, 0.1 mM PMSF), lysed by sonication, and clarified at 30,000g for 30 min at 4 °C. Clarified lysate was then incubated with amylose resin for 1 h and washed with high salt buffer (50 mM Tris HCl pH 7.4, 0.1 mM TCEP, 500 mM NaCl, 5% glycerol) and then low salt buffer (50 mM Tris HCl pH 7.4, 0.1 mM TCEP, 150 mM NaCl, 5% glycerol). His-MBP-tagged *TcPINK1* was eluted from the resin using low salt buffer containing 12 mM maltose.

¹⁵N-Labeled ubiquitin was expressed in 2xYT media from Rosetta 2(DE3) pLysS *E. coli* cells using the high-cell-density expression method adapted from Marley *et al.*⁴⁰ Once cultures reached an OD₆₀₀ of 0.8, cells were concentrated (4×) and transferred into M9 media. Cells were incubated for 1 h at 37 °C to allow discharge of unlabeled metabolites and then supplemented with 20% w/v glucose and 0.5 g of ¹⁵NH₄Cl, and induced with 800 μ M IPTG followed by 4 h expression at 37 °C. Cells were harvested and purified as previously reported.

Generation of pSer65-Ubiquitin.

Ub, ¹⁵N-Ub, or Lys63 di-Ub (5 mg) was resuspended in 50 mM Tris HCl pH 7.4, 5 mM MgCl₂, and 0.8 mM ATP. Phosphorylation was initiated by adding 5 μ M *TcPINK1* and incubated overnight at 30 °C. The reaction was quenched with 10% v/v ammonium acetate and buffer exchanged in 20 mM Tris pH 8.7. Phosphorylated Ub was further separated using anion exchange with running buffer A (20 mM Tris pH 8.7) and buffer B (50 mM Tris pH 7.4) using a 0–60% gradient over 7 CV.

Ubiquitination/Deubiquitination Assays.

Ubiquitination/deubiquitination assays were performed by mixing 150 nM E1, 1 μ M UBE2L3, 1.3 μ M Parkin, 30 μ M ubiquitin, USP30 (as indicated), and 2 mM ATP in 50 mM Tris HCl pH 7.4, 5 mM MgCl₂. Ubiquitination was initiated by adding 0.5 μ M *TcPINK1*. The reaction took place for indicated time points at 30 °C and was quenched by adding 6× Laemmli buffer (for Western blotting) or flash frozen (for mass spectrometry).

Parkin Pulldown Assay.

In vitro ubiquitination reactions were flash frozen and diluted into binding buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.05% IGEPAL) and incubated at room temperature for 2 h with Ni-NTA resin. Following four washes with minimal buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl) and water, the resin was resuspended in TEV cleavage buffer (50 mM Tris HCl pH 7.4, 0.5 mM TCEP). Ubiquitinated Parkin was released from Ni-NTA resin using TEV protease overnight at 4 °C to remove the His6-SUMO tag.

Minimal Trypsin Digestion.

For mass spectrometry, ubiquitinated Parkin samples were buffer exchanged into 25 mM ammonium bicarbonate, whereas *in vitro* ubiquitination/deubiquitination reaction products were flash frozen at indicated time points. Using an empirically determined ratio of trypsin to ubiquitin (1:150 or 30 μ L of total reaction mixture), all samples were digested under nondenaturing conditions for 2 h at 37 °C with trypsin (0.5 μ g).^{35,41} Trypsin was deactivated with 10% v/v acetic acid and incubated at 4 °C for 30 min, and the resulting mixtures were dialyzed into water using Slide-A-lyzer MINI dialysis units with a 3.5 kDa MWCO (ThermoFisher). All samples were then dried using a speed vac and resuspended into 20 μ L of 1% formic acid.

Linear Range and Reproducibility of N-15 Ub Standards.

15N-Ub and 15N-pSer65 Ub mixtures were prepared at 25, 50, 150, 250, and 500 fmol/ μ L in a constant background of 75 ng/ μ L of unlabeled Ub and pSer65 Ub in 1% formic acid. On-column injections (2 μ L) were performed in a randomized order.

Middle-Down Mass Spectrometry.

Samples were injected (5 μ L) and separated on an Easy nLC 1000 UHPLC equipped with a homemade 15 cm nanoLC column (ProntoSIL C4 5 μ m 300A, NanoLCMS Solutions) using a linear gradient of 0 to 5% B over 5 min, 5 to 70% B over 20 min, and 70 to 90% over 10 min (solvent A: 0.1% FA in water, solvent B: 0.1% FA in ACN) using a flow rate of 300 nL/min. The resolution of the Orbitrap Fusion was set at 60,000 with an AGC target of 4.0×10^5 and a maximum injection time of 200 ms. For tandem mass spectrometry (MS/MS) using ETD, individual charge states of the protein molecular ions were isolated and dissociated by ETD using 10 ms reaction time, 2.0×10^5 reagent ion target, and 10% supplemental collisional induced dissociation. The resolution of the Orbitrap Fusion was set at 60,000 with an isolation window of 1.7 m/z , an AGC target of 5.0×10^4 , and maximum injection time of 500 ms. All spectra were processed with MASH Suite Software using a signal-to-noise (S/N) threshold of 3 and a fit factor of 70% and then validated manually.^{42,43} Percentages correspond to the relative quantification values between the charge states of 11+ for all five species: Ub₁₋₇₄, pSer65 Ub₁₋₇₄, 1xdiGly-Ub₁₋₇₄, pSer65 1xdiGly-Ub₁₋₇₄, and 2xdiGly-Ub₁₋₇₄ after normalization with ¹⁵N-labeled standards.

RESULTS AND DISCUSSION

Middle-Down MS Analysis of Parkin-Mediated Chain Assembly.

Ubiquitination reactions with Parkin and PINK1 were monitored by Western blot analysis using α -Ub and α -pSer65-Ub antibodies. At the earliest time point (5 min), high molecular weight (HMW) Ub conjugates corresponding to autoubiquitinated products and unanchored ubiquitin chains can already be observed (Figure 1A). By 1 h, the formation of HMW conjugates has nearly reached its plateau. Immunoblotting with the α -pSer65-Ub antibody shows that pSer65-Ub is also incorporated into the HMW conjugates within a similar time frame.

We then availed middle-down MS to gain more insight into the architecture of chains produced by Parkin. Crude reaction mixtures were trypsinized under native conditions to generate Ub₁₋₇₄ variants. Subsequent intact MS analysis shows that mono-Ub (Ub₁₋₇₄), not surprisingly, represents the bulk of the Ub species (Figure 1B). Nevertheless, unphosphorylated (diGly-Ub₁₋₇₄) and phosphorylated (diGly-pSer65-Ub₁₋₇₄) chains can still be observed along with branch points (2xdiGly-Ub₁₋₇₄). What is rather intriguing is the ratio of branching (2xdiGly-Ub₁₋₇₄) to the unbranched portion (diGly-Ub₁₋₇₄) of a chain. Using mixtures of linkage specific enzymes to assemble branched chains, we typically measure ratios of 0.1–0.2. With Parkin, the ratio is close to 0.5, suggesting chain branching is extensive.

Previous work has shown that Parkin is unable to incorporate pSer65-Ub into chains when pSer65-Ub is the only source of Ub.^{34,44} Our middle-down data reveal that subunits within a chain are phosphorylated when PINK1 can act on Ub anytime during the chain assembly process. To determine whether phosphorylation occurs on a particular chain type, we performed electron transfer dissociation (ETD) MS/MS analysis on the diGly-pSer65-Ub₁₋₇₄ species. The ETD data fit nicely with the diGly motif on Lys63 (Figure 1C and Figure S1B), suggesting PINK1 phosphorylates internal subunits of Lys63 chains. These results are consistent with data showing that PINK1 phosphorylates each subunit of well-defined Lys63 chains at a faster rate than other chain types.

Isotopically Labeled Ub Standards for Absolute Quantitation.

In the absence of internal standards, it is difficult to determine how much unmodified and diGly-modified pSer65-Ub is generated relative to the unphosphorylated forms of Ub due to the possibility that pSer65-Ub and Ub have different ionization efficiencies. To assess this, we prepared ¹⁵N-labeled Ub and pSer65-Ub with the expectation that these reagents would also allow us to quantify the abundance of each species (Figure S2A).

In a mixture containing a constant background of light (¹⁴N-labeled) Ub and pSer65-Ub (Figure S2B), we examined the response to different concentrations of ¹⁵N-labeled Ub and pSer65-Ub. LC traces show that all four Ub species have similar retention times using a 15 cm C4 reverse-phase column (Figure 2A). The phosphate group does indeed cause ion suppression, as the total ion count is 5-fold more sensitive to increasing concentrations of ¹⁵N-labeled Ub compared to ¹⁵N-labeled pSer65-Ub (Figure 2B). Despite these differences,

a linear response is observed for both ^{15}N -labeled Ub and pSer65-Ub, indicating the two heavy variants can serve as internal standards.

Using synthetic Ub chains, we have already shown that the relative abundance of each truncated Ub variant, as measured by middle-down MS, reflects the ratios of different segments of a chain, that is, the distal cap, the unbranched segment, and the branch point. We confirmed this observation again using Lys63 Ub dimers (di-Ub); intact MS shows a 1:1 ratio of Ub₁₋₇₄ (the distal subunit) and diGly-Ub₁₋₇₄ (the proximal subunit). We then asked whether the ratio of unmodified to diGly-modified pSer65-Ub measured by intact MS reflects the actual ratio of each phosphorylated species. Using PINK1 to completely phosphorylate Lys63 di-Ub, we found that diGly-pSer65-Ub₁₋₇₄ does ionize slightly better than pSer65-Ub₁₋₇₄, but the ratio is close to 1:1, indicating that a single standard can report on the relative abundance of each species (Figure 2C and Figure S2D).

Quantitation of Parkin Autoubiquitination.

With the ability to quantitate middle-down MS data, we sought to measure the abundance of Ub and pSer65-Ub in their unmodified and modified forms. Instead of analyzing the bulk reaction, we chose to follow the assembly of chains directly on Parkin. Autoubiquitination of Parkin is often used to assess activity,¹⁸ and we wanted to minimize the impact of unincorporated mono-Ub on the analysis of chain architecture.

To isolate autoubiquitinated Parkin from reaction mixtures, we performed a two-step purification protocol using Ni-NTA chromatography (Figure 3A). Unanchored Ub chains along with mono-Ub were removed from the HMW conjugates containing Parkin in the first Ni-NTA chromatographic step (Figure 3B). To ensure we only examined chains directly tethered to Parkin, the 6xHis-SUMO tag originally fused to the N-terminus of Parkin was cleaved by TEV, and the resulting autoubiquitinated Parkin products were released from Ni-NTA resin. Immunoblotting with the α -Ub and α -Parkin antibodies shows the elution contains a substantial amount of HMW Ub conjugates along with Parkin.

Autoubiquitinated Parkin was then isolated at different time points and analyzed by quantitative middle-down MS (Figure 3C). After minimal trypsinolysis, ^{15}N -labeled Ub and pSer65-Ub standards were spiked into each sample to quantify the abundance of each Ub species. The Ub species were then separated from other peptides using a C4 reverse-phase column. As observed with purified light and heavy Ub variants, there is a slight difference in retention time between unphosphorylated and phosphorylated species ($RT = 0.2$ min) (Figure S3A). Mass spectra were initially processed using Xcalibur Qual Browser v4.2 (Figure S3B). The total ion count of each Ub variant in the 11+ charge state was integrated using a mass tolerance of 20 ppm with Gaussian smoothing of seven points and mass precision of four decimal points. Using this approach, quantitation of unmodified and modified pSer65-Ub is compounded by low charge state peptides (Figure 3C). To overcome this problem, we calculated the relative abundance of Ub variants by summing the peak heights of the most abundant isotopomer, which affords a total ion count. This was achieved using the quantitation node in the Mash Suite program (Figure S3B).^{42,43}

As the autoubiquitination reaction proceeds, the amount of Ub attached to Parkin increases. This increase leads to an overall decrease in ionization of all Ub species (Figure 3D). By adding ^{15}N -labeled Ub and pSer65-Ub standards to samples from individual time points, we are able to accurately quantify the relative abundance of all Ub variants. Each of the unphosphorylated Ub species (*i.e.*, Ub₁₋₇₄, diGly-Ub₁₋₇₄, and 2xdiGly-Ub₁₋₇₄) is observed within the first 5 min of the reaction (Figure 3C,E). After 45 min, Ub₁₋₇₄ remains the major species, indicating that Parkin primarily monoubiquitinates itself; however, diGly-Ub₁₋₇₄ and 2xdiGly-Ub₁₋₇₄ represent ~24 and 9% of the total Ub population, respectively. According to ETD analysis, branch points are composed of Lys6 and Lys48 linkages similar to the bulk reaction (Figure S3C). These results suggest that Lys6/Lys48 branch points are introduced concomitantly with the rest of the chain.

Quantification of phosphorylated Ub variants reveals a slight delay in the formation of diGly-modified pSer65-Ub relative to the unmodified form (Figure 3F). As observed with the unphosphorylated Ub species, unmodified pSer65-Ub is detected at the earliest time point (5 min) and reaches 11% of the total Ub population by 45 min. DiGly-modified pSer65-Ub is not detected until the 15 min time point and only represents 2% of the total Ub population at 45 min of autoubiquitination. These data indicate that Parkin modifies itself with pSer65-Ub, but very little of the phosphorylated species is incorporated into chains. These results are entirely consistent with recent reports that utilized bottom-up methods to determine the architecture of chains built by PINK1 and Parkin both *in vitro* and in cells such as HeLa and iNeurons.^{15,32-34,45}

USP30 Changes the Landscape of Parkin-Derived Ubiquitin Chains.

USP30 is an integral MOM protein thought to oppose the activities of PINK1 and Parkin and therefore acts as a brake during mitophagy.³⁸ Biochemical studies have shown that USP30 prefers to cleave Lys6 Ub chains but is inhibited upon PINK1-mediated phosphorylation of the distal subunit, suggesting USP30 activity diminishes as PINK1 and Parkin reach full activation. Parkin does not just assemble Lys6 chains; Lys11, Lys48, and Lys63 linkages are all products of Parkin.^{15,32} Yet, how these different chain architectures affect USP30s activity remains unclear.

We therefore monitored PINK1 and Parkin-dependent chain assembly reactions in the presence of different concentrations of USP30. At low concentrations of USP30 (0.2 μM), chain disassembly is only observed at later time points (60 and 120 min) and immunoblotting with the α -pSer65-Ub antibody reveals very little cleavage of the phosphorylated, HMW conjugates (Figure 4A). At higher concentrations (6 μM), chain disassembly is much more robust, but the disassembly of phosphorylated conjugates is still minimal and is only observed at later time points.

Quantitative middle-down MS analysis provides a more detailed look at what is happening during chain assembly in the presence of USP30. Congruent with its ability to dismantle chains, the Ub₁₋₇₄/diGly-Ub₁₋₇₄ ratio shifts from ~3:1 in the absence of USP30 to approximately 6:1 in its presence after 1 h (Figure 4B,C). The most significant effect, however, is on the relative abundance of branch points. After 1 h, Lys6/Lys48 branch points (2xdiGly-Ub₁₋₇₄) comprise 4% of the total Ub population in the absence of USP30. When

USP30 is present, branch points are nearly undetectable. These results suggest that Lys6/Lys48 branched chains are particularly susceptible to cleavage by USP30.

We also noticed that the disassembly of unphosphorylated chains by USP30 leads to an enrichment of unmodified pSer65-Ub. This can be seen by comparing the relative abundance of Ub₁₋₇₄ and pSer65-Ub₁₋₇₄ at the 30 min time point; the presence of USP30 causes the latter to become the major species (Tables S2 and S3). These data can be rationalized by a scenario in which USP30 continuously replenishes the pool of mono-Ub, providing PINK1 with more substrate.

CONCLUSION

In this study, we report on the quantitative middle-down MS analysis of ubiquitin chains produced by Parkin in the presence and absence of the deubiquitinase USP30. Quantification of both Ub and pSer65-Ub in their unmodified and modified forms is achieved using uniformly, ¹⁵N-labeled Ub and pSer65-Ub internal standards. Our results show that Parkin is a prolific chain branching E3 ligase capable of building Lys6/Lys48 branch points. We find that Parkin also attaches pSer65-Ub to itself in the form of a single Ub moiety or at the distal end of a chain. Additionally, we find that when pSer65-Ub is incorporated into a Ub chain, it does so with a Lys63 linkage. In the presence of USP30, branched chains are preferentially lost relative to the ensemble of unbranched chains. Thus, quantitative middle-down MS can be used to inform on the architecture of chains produced by Parkin and how these chains are processed by deubiquitinases such as USP30.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was funded by the NIH (RO1GM110543 to E.R.S.) and a NSF Graduate Research Fellowship (GRFP1451512 to K.K.D.). The data described herein were acquired on an Orbitrap Fusion mass spectrometer funded by the NIH grant 1S10OD010645-01A1.00058.

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Figure 1.

Ubiquitin chain assembly by Parkin. (A) Time course analysis of ubiquitination reactions with Parkin (1.3 μ M) and PINK1 (0.5 μ M). (B) Middle-down MS analysis of a 60 min Parkin reaction after minimal trypsinolysis with UBE2L3 (1 μ M) as E2. The spectrum corresponds to the Ub¹¹⁺ charge state. (C) ETD analysis (c and z* ions) of diGly-pSer65-Ub₁₋₇₄ mapped onto the Ub sequence containing a di-Gly modification at Lys63 and phosphorylation at S65. Red circles represent theoretical isotopic abundance distribution of isotopomer peaks. Calc'd: calculated monoisotopic weight; exp't'l: experimental monoisotopic weight.

Figure 2.

Ionization efficiency of ubiquitin species. (A) Extracted ion chromatograms of Ub, pSerUb, ¹⁵N-Ub, and ¹⁵N-pSer Ub shown on the same relative scale with retention times for each. (B) Linear response curves for ¹⁵N-Ub (top) and ¹⁵N-pSer Ub (bottom) shown with R^2 and slope values determined for each linear fit. The solid black lines represent the 95% confidence interval for the average fits of two independent experiments. (C) Middle-down MS of Lys63 Ub₂ and pSer65 Lys63 Ub₂ with percentages based on the relative abundance of each Ub variant. The spectra correspond to the Ub¹⁺ charge state.

Figure 3.

Autoubiquitination of Parkin. (A) General scheme for the assembly and isolation of autoubiquitinated Parkin. Captured Parkin was subjected to minimal trypsinolysis under nondenaturing conditions to yield a variety of Ub species. (B) Western blot analysis of the pull-down from crude reactions using Ni-NTA resin. Autoubiquitinated Parkin was released by incubation with TEV protease overnight. (C) Middle-down MS analysis of autoubiquitinated Parkin after minimal trypsinolysis. The spectra correspond to the Ub¹¹⁺ charge state and percentages are normalized against the ¹⁵N-Ub and ¹⁵N-pSer65 Ub standards. (D) Ionization efficiency of ¹⁵N-Ub and ¹⁵N-pSer65 Ub standards plotted in reference to total Ub detected during Parkin autoubiquitination time course. Quantitation of (E) unphosphorylated and (F) phosphorylated Ub variants produced during Parkin autoubiquitination time course.

Figure 4. Parkin and USP30 ubiquitination reactions. (A) Western blot analysis of Ub chain assembly by Parkin (1.3 μ M) or disassembly by varying concentrations of USP30 against the Ub and phosphoUb antibodies. Middle-down MS analysis of a crude Parkin (1.3 μ M) reactions (B) without or (C) with the presence of USP30 (1.3 μ M). The spectra correspond to the Ub¹¹⁺ charge state and percentages are normalized against the ¹⁵N-Ub and ¹⁵N-pSer Ub standards.