Conformational stabilization of Optineurin by the dynamic interaction of linear polyubiquitin

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Abstract
Optineurin produces intracellular multi-functions involving autophagy, vesicular trafficking, and negative regulation of inflammation signaling through interaction with various proteins such as ATG8/LC3, Rab8, and polyubiquitin. Optineurin is a component of cytoplasmic inclusion bodies (IBs) in motor neurons from amyotrophic lateral sclerosis (ALS), and its mutation E478G, has been identified in patients with ALS. However, the mechanism by which polyubiquitin binding modulates the interaction partners of OPTN and ALS-associated IB formation is still unclear. To address this issue, we analyzed the interaction of Optineurin with Rab8 and LC3 in the absence and presence of linear polyubiquitin chains using fluorescence cross-correlation spectroscopy and IB formation efficiency of the E478G mutant of Optineurin during Rab8 depletion using fluorescence microscopy. Here, we hypothesize that linear polyubiquitin binding to Optineurin dynamically induces LC3 association and Rab8 dissociation, likely through a conformational change of Optineurin, and the dynamic conformational change may prevent the aggregate formation of mutant Optineurin.
1. Introduction

Optineurin (optic neuropathy inducing; OPTN, also called FIP2 or NRP), a 577 amino acid protein encoded by the *OPTN* gene, has several coiled-coil domains (CCs), leucine zipper, zinc finger, and ubiquitin (Ub)-associating region (UBAN), and mediates various functions by interacting with many different proteins, such as small GTP-binding Ras-related protein in brain 8 (Rab8), autophagy-related protein 8 (ATG8; also known as LC3 in mammals), Huntingtin, and Ub. Since there is still no information on the three-dimensional whole structure of OPTN, the position of the CCs varies among reports. According to a secondary structure prediction, OPTN has at least two CCs (CC1 and CC2; amino acids 26–119 and 230–445, respectively) [1]. A CC structure of amino acids 27–105, a fragment of human OPTN with TANK-binding kinase 1 (TBK1), was identified using X-ray crystallography [2], indicating that the above prediction is not irrelevant. No experimental evidence for putative CC2 structures has yet been fully elucidated. UBAN of mouse OPTN (amino acids 417–513 corresponding to 414–510 in human sequence) with Ub dimers also forms a CC structure [3]. Huntingtin and Myosin VI, classically well-known interacting partners of OPTN, are known to bind to the C-terminal side of putative CC2 and the region containing UBAN [1,4]; thus, the UBAN of OPTN would be continuously linked to CC2. CC1 and CC2 are linked to a putative disordered region. Both Rab8- and LC3-interacting regions in OPTN (amino acids 141–209 and 169–209, respectively) are located in or include the linker region between CC1 and CC2 and are separate from UBAN [1,4].

Regarding Rab8, in humans and mice, there are two isoforms with approximately 80% homology (Rab8A and Rab8B), which have a differential expression pattern [5]. Because of the high similarity between the two subtypes, it is difficult to determine the distinct function of its subtypes, Rab8A and Rab8B; however, several functional and expression differences have been determined using knockout mice [6]. Rab8A is ubiquitously expressed, whereas Rab8B is limited to the brain, spleen, and testes [5,7]. Rab8B knockout mice did not show an overt phenotype, whereas Rab8A knockout mice showed defects in the localization of apical marker proteins in intestinal cells [8]. Rab8A and Rab8B double knockout mice and Rab8A knockout mice exhibited short survival rates [6]. Accordingly, Rab8A is considered to carry a primary function of Rab8, and Rab8B would be an auxiliary and redundant subtype in many tissues and cells.

Autophagy is a degradation system in eukaryotic cells that targets specific cargo, such as aggregated proteins, damaged mitochondria, and invading pathogens through autophagy receptors (OPTN, p62/SQSTM1, NDP52, etc.) [9]. LC3 is known to be a typical marker and a critical player in autophagy activity because the cytosolic isoform of LC3 (LC3-I) is converted to its lipid-modified and membrane-bound isoform (LC3-II), which promotes autophagy activity [10]. OPTN harbors both UBAN and the LC3-interacting region (LIR), similar to other autophagy receptors such as p62/SQSTM1 and NDP52 [11,12]. Various types of polyubiquitin chains and their ubiquitination states...
have been identified (e.g., linear, K48-, and K63-linked polyUb chains and other linked states) [13]. OPTN preferentially binds to linear and K63-linked polyUb chains, but not to K48-linked chains [14]. Such linear and K63-linked polyUb chains decorate the infected bacteria and serve various downstream signaling pathways involving xenophagy and NF-κB activation [13]. Because of the interacting features of OPTN, the role of OPTN in post-Golgi vesicular trafficking and selective autophagy, including mitophagy and xenophagy, has been the focus of research [12,15]. Rab8 and LC3 are key interactors of OPTN for post-Golgi trafficking and autophagy, respectively [12,16].

Mutations in OPTN that lead to amino acid substitutions are found to cause several genetic diseases, including amyotrophic lateral sclerosis (ALS) and primary open-angle glaucoma (POAG) [15,17]. In motor neurons from ALS patients, Ub- and TDP-43-positive OPTN inclusion bodies (IBs) with the E478G mutation have been identified [18]. The E478G mutant of OPTN forms IBs in the cytoplasm of mammalian cells [19,20]. The ALS-associated Q398X and E478G mutants of OPTN, which impair Ub-binding activity, cause defects in vesicular trafficking [21]. Autophagosome- or trafficking vesicle-associated foci of OPTN are usually observed [2,18,21]. On the other hand, misfolded or denatured proteins in cells often form aggregates, which then accumulate in the IBs [22,23]. Such emerging aggregates with aging are thought to be toxic and cause neurodegeneration [24].

However, it is still unknown how the polyUb signaling pathway is involved in the interaction between OPTN and Rab8. Moreover, it is also unclear why the E478G mutant of OPTN forms IBs in the cell. Therefore, we set out to determine how the interaction change between Rab8 and OPTN by polyUb addition affects the conformation of OPTN using fluorescence cross-correlation spectroscopy (FCCS) and fluorescence microscopy.

Methods

Preparation of plasmid DNA

The plasmids encoding eGFP-tagged wild type or ALS-linked E478G-mutant of optineurin (G-OPTN-WT or EG) were used as previously reported [19]. The cDNA of Rab8A was obtained from Addgene (#24898; Watertown, MA, USA). The PCR-amplified coding region of Rab8A was cut into XhoI and BamHI sites and inserted into pEGFP-C1 (R-Rab8). The coding region of LC3 cut from ptPLC3 [25] was inserted into pmCherry-C1 (R-LC3). Plasmids encoding mCherry-eGFP tandem dimers, mCherry monomers, or eGFP monomers for FCCS were used as previously reported [26].

Cell culture and transfection

Mouse neuroblastoma Neuro2a cells were maintained as previously reported [26]. For cell lysis for FCCS and cell fixation for immunofluorescence staining, 2.0 × 10^5 cells were grown in a 3.5 cm dish (#150318, Thermo Fisher Scientific, Waltham, MA) for 16 h before transfection. The plasmids coding
for G-OPTN-WT or -EK (0.5 μg) and R-Rab8 or R-LC3 (0.5 μg) were transfected into cells using Lipofectamine 2000 (2.5 μL) (Thermo Fisher Scientific). After incubation of the cells for 24 h, subsequent experiments were performed. For Rab8A knockdown, 1.0 × 10^5 cells were grown in a 3.5 cm dish (#150318, Thermo Fisher Scientific) for 24 h before transfection. The duplex of small interfering RNA (siRNA) for Rab8A (5’-GACAGGUGUCCAAGGAACGtt-3’ and 5’-CGUUCCUUGGACACCCUGUtt-3’) was synthesized by GeneDesign Inc. (Osaka, Japan). The siRNA (25 pmol) for Rab8A or non-specific target as a negative control (#AM4635; Thermo Fisher Scientific) was transfected into the cells with 2.5 μL of Lipofectamine RNAi MAX (Thermo Fisher Scientific).

**Fluorescence cross-correlation spectroscopy (FCCS)**

Cells were lysed in a lysis buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The soluble fraction was recovered by centrifugation at 20,400 × g for 5 min at 4 °C, FCCS measurements were performed using a Confocor 3 system combined with an LSM 510 META (Carl Zeiss, Jena, Germany) through a C-Apochromat 40×/1.2 NA Korr UV-VIS-IR water immersion objective (Carl Zeiss) as reported previously [26]. Measurements were performed in a cover-glass chamber (#155411, Thermo Fisher Scientific, Waltham, MA) in the absence or presence of 2.5 μM linearly linked 6-mer polyUb (M1-Ub; #D4220; Ubiquitin-Proteasome Biotechnologies, Aurora, CO).

**Immunofluorescence staining and confocal microscopy**

Neuro2a cells were fixed in methanol (-20 °C). Cells were blocked in 5% normal goat serum (Wako, Osaka, Japan) and 20% glycerol in PBS. Mouse monoclonal antibodies against Rab8 (#610844, BD Biosciences, San Jose, CA) or Alexa Fluor 488-conjugated rat monoclonal anti-GFP antibody to enhance GFP fluorescence (#D153-A48, MBL, Nagoya, Japan) were diluted in Can Get Signal immunostain Solution A (NKB-501; TOYOBO, Osaka, Japan). For counterstaining of OPTN-E478G IBs instead of Rab8, mouse monoclonal anti-GFP antibody (#GF200, Nacalai Tesque, Kyoto, Japan) was used. Goat polyclonal anti-mouse IgG antibody conjugated with Alexa Fluor 594 (#A-11005, Thermo Fisher) was used. The cells were mounted with ProLong Glass (Thermo Fisher). Images were acquired using a confocal laser scanning microscope (LSM510 META, Carl Zeiss) with a Plan-Apochromat 63×/1.4 NA oil immersion objective with Immersol 518F immersion oil (refractive index of 1.518 at 25 °C; Carl Zeiss).

**Results & Discussion**

*Linear polyubiquitin is a modulator for protein interaction of OPTN*

To determine the interactions between OPTN and Rab8 or LC3 in Neuro2a cell lysate, we used
fluorescence cross-correlation spectroscopy (FCCS), which detects the interaction of fluorescent molecules from the simultaneity of diffusion of two different colored molecules in the confocal detection volume with single-molecule sensitivity. Tandem dimers of mCherry and eGFP as a positive control (PC) and mixtures of mCherry and eGFP monomers as a negative control (NC) showed high and low relative cross-correlation amplitude (RCA), respectively (lanes 1 and 2 in Figure 1, A and B). The low but slightly positive RCA of the NC sample is due to the spectral crosstalk between two color detection channels, and it indicates the lowest limit of interaction detection in the FCCS condition. Positive RCA was observed for both eGFP-tagged wild-type OPTN (G-OPTN-WT) and E478G mutant of OPTN (G-OPTN-EG) with mCherry-tagged Rab8A (R-Rab8) (lanes 3 and 5, Figure 1A). To determine whether the interaction between OPTN and Rab8 could be changed by polyUb, non-fluorescent linear-linked polyUb (M1-Ub) was added to the cell lysate containing G-OPTN and R-Rab8. RCA in G-OPTN-WT was decreased by M1-Ub addition, but not in G-OPTN-EG (Lanes 4 and 6, Figure 1A), suggesting that M1-Ub binding promotes the dissociation of Rab8 from OPTN via its UBAN domain. Moreover, the decreased RCA between wild-type OPTN and Rab8 was still positive (lane 4, Figure 1A), indicating that M1-Ub could not completely eliminate the dissociation between OPTN and Rab8.

Next, we examined whether the interaction between OPTN and LC3 was changed by M1-Ub addition using FCCS. Positive RCA was observed for both G-OPTN-WT and -EG with R-LC3 without M1-Ub addition (lanes 3 and 5, Figure 1B), suggesting that OPTN and LC3 spontaneously interacted without polyUb chain uptake. RCA between G-OPTN-WT and R-LC3 was increased by M1-Ub addition; however, RCA between G-OPTN-EG and R-LC3 was not (Lanes 4 and 6, Figure 1B), suggesting that M1-Ub accelerates the association between OPTN and LC3 via its UBAN domain. The inverse relationship of RCA between LC3 and Rab8 after M1-Ub addition suggests that polyUb binding may modulate OPTN-interacting partners. Because the Rab8- and LC3-binding region in OPTN is located far from the UBAN in the primary structure, the interaction of Rab8 or LC3 with poly-Ub against OPTN is not a simple competitive inhibition in the UBAN. The disordered region between CC1 and CC2 in the OPTN contributes to making a flexible structure and can be twisted and turned through disordered regions. Rab8- or LC3-interacting regions are located in the disordered region of the OPTN. The binding of Ub to UBAN may induce a conformational change in OPTN, which in turn may affect the configuration of the Rab8- and LC3-binding regions. Accordingly, the interaction between OPTN and poly-Ub likely leads to a conformational change in OPTN, which then dynamically modulates the interaction between Rab8 and LC3 against OPTN.

To determine the oligomerization state of OPTN, counts per molecule (CPM), which indicates the mean brightness of a single particle, were analyzed. CPMs of GFP-OPTN-WT or -EG that were co-expressed with R-Rab8 did not increase in the absence or presence of M1-Ub, suggesting that OPTN oligomerization is not promoted by Rab8 overexpression and subsequent addition of M1-Ub
(lanes 1–4, Figure 1C). In contrast, CPMs of GFP-OPTN-WT or -EG that were co-expressed with R-LC3 were increased even before M1-Ub addition in the cell lysate (lanes 1 and 3, Figure 1D), suggesting that R-LC3 overexpression promotes OPTN oligomerization. A possible explanation is that, because LC3 can form aggregates independent of autophagy [27], soluble oligomers/aggregates of LC3 promoted by its overexpression may sequester OPTN, resulting in an increase in OPTN oligomers. Next, M1-Ub addition in cell lysate increased the CPM of GFP-OPTN-WT but not dramatically in that of GFP-OPTN-EG (Lanes 2 & 4, Figure 1D), suggesting that M1-Ub may lead to complex formation of OPTN and LC3 through a conformational change of OPTN via polyUb interaction.

Accordingly, M1-Ub modulates the interaction state of OPTN, at least with Rab8 and LC3, possibly through the conformational change of OPTN. Since M1-Ub, produced by the linear ubiquitin chain assembly complex (LUBAC), is known to decorate infected bacteria to induce an autophagy system called xenophagy to initiate an immune response and its degradation [28,29], such conformational changes and interaction switching of OPTN may play an important role in xenophagy progression by dissociating OPTN from Rab8 and efficiently using OPTN for xenophagy. Moreover, the maintained interaction of OPTN-EG with Rab8 even in M1-Ub increased. However, because Golgi fragmentation was frequently observed in neurons from an ALS patient bearing the E478G mutation [30], the persistence of the interaction between OPTN and Rab8 seems to be a steady state but may dysregulate vesicular trafficking and membrane transport in the Golgi and/or other membrane trafficking pathways, leading to disturbance or degeneration of neurons during aging.

\textit{Rab8 accumulates in the aggregate of ALS-linked mutant of OPTN}

Next, to determine the role of OPTN-EG and Rab8, we observed colocalization between the EG mutant of OPTN and Rab8 in Neuro2a cells using fluorescence microscopy. Transiently expressed G-OPTN-EG and R-Rab8 were colocalized in the inclusion body-like structures (IBs) in the cytoplasm, whereas a fluorescent tag as a control was not (Figure 2A). This indicates that R-Rab8 accumulates in IBs with the EG mutant of OPTN. Next, to determine whether endogenous Rab8 accumulates in the IBs of the EG mutant of OPTN, immunofluorescence microscopy was performed. A portion of the wild-type OPTN foci was colocalized with Rab8, but many of them were not (Figure 2B, top, and 2C, left). Rab8 was observed to be localized in the border region of the IBs of the EG mutant of OPTN (Figure 2B, bottom, and 2C, right). The boundary staining pattern using anti-Rab8 antibody was the same as that using the anti-GFP antibody in the OPTN-EG IBs (Sup. Fig. 1). Thus, the OPTN-EG IBs may have a dense structure in which antibodies cannot be soaked. Moreover, the green and magenta signals in the edge region of the IB with high intensity overlapped perfectly (Figure 2B, arrowheads). Accordingly, Rab8 was located inside the IBs of the OPTN-EG.

\textit{Rab8 knockdown ameliorates aggregates of ALS-linked mutant of OPTN}
To elucidate the functional role of Rab8 in OPTN IB formation, we knocked down Rab8 in Neuro2a cells. Since no overt phenotype in Rab8B-knockout mice [6] and ubiquitous expression of Rab8A have been reported [7], Rab8A was selected as the knockdown target. The amount of Rab8A in Neuro2a cells transfected with siRNA for Rab8A decreased to approximately 70% compared to the non-specific (NS) siRNA-transfected control (Figure 3A). The population of cells containing the IBs of OPTN-EG was significantly decreased in Rab8A-knockdown cells (Figure 3B). A possible explanation is that Rab8A may assist the accumulation of the E478G mutant of OPTN in the IBs and/or maintain a stable conformation to prevent aggregation of OPTN outside the IBs. Molecular chaperones are known to prevent aggregation and IB formation [31,32]. Rab8 has an opposite role to that of molecular chaperones against OPTN.

The concentration of polyUb is thought to change dynamically depending on the signaling pathway and the related activity of Ub ligases and deubiquitinating enzymes (DUBs) [13]. Thus, the interaction between OPTN-WT and Rab8 may dynamically change depending on the linear polyUb concentration. This dynamic interaction between OPTN-WT and Rab8 contributes to maintaining the interactome of OPTN with various other interacting proteins, resulting in the prevention of abnormal OPTN accumulation. Typically, the interaction between OPTN and LC3 may play a role in preventing the aggregation of OPTN, and thus, OPTN forms a functional complex as an autophagy receptor, leading to autophagy with LC3 (Figure 4A). In contrast, the conformational change of OPTN-EG would not be evoked because of impaired Rab8 dissociation by polyUb binding, resulting in the aggregation of OPTN in the binding to Rab8 (Figure 4B). In the absence of Rab8, the conformational change of the E478G mutant of OPTN occurs or is induced by other proteins. As a result, such modulation in OPTN-EG affected aggregate formation and accumulation of OPTN in the IBs (Figure 4C). Therefore, Rab8 could assist in the accumulation of OPTN by prolonging the conformation of OPTN. Although OPTN is thought to be an effector protein for Rab8 [4,16], our results suggest that Rab8 may be a modifier that maintains the OPTN oligomerization state.

We provide a new hypothesis that the dynamic conformational change of OPTN regulated by interacting proteins and linear polyUb maintains stability and prevents aggregation of OPTN. These findings support the potential to reveal the physiological function and mechanism of ALS-linked IB formation of OPTN in the cytoplasm. Moreover, FCCS analysis of cell lysates by the addition of proteins, peptides, and small molecules, such as polyUb, is a powerful tool for analyzing the interaction modulation between multiple proteins.

Author contributions
Conceived and designed the experiments: AK. Performed the experiments: AK and RN. Analyzed the data: AK, RN, and MK. Wrote the manuscript: AK and MK. All the authors agree with the publication of this paper.
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Figure legends

Figure 1. Interaction analysis of GFP-OPTN with mCherry-Rab8A or mCherry-LC3 by Linear polyubiquitin chain addition using fluorescence cross-correlation spectroscopy.
Relative cross-correlation amplitude (RCA) plot (A & B) and counts per molecule (CPM) plot (C & D) of GFP-OPTN with mCherry-Rab8A (A & C) or mCherry-LC3 (B & D) in the absence (-) or presence (+) of linear polyUb chain (M1-Ub). PC, NC, WT, and EG denote the mCherry-GFP tandem dimer as a positive control, the mixture of GFP and mCherry monomers as a negative control, wild-type OPTN, and ALS-linked E478G mutant of OPTN, respectively. As RCA with both Rab8A and LC3 in (A) and (B) are trials on the same day, the values of PC and NC are the same set. Bottom illustrates in (A) and (B) a representative model for the interaction change of OPTN with Rab8A or LC3 by M1-Ub addition. Bars indicate the mean and SD; dots indicate the values of independent trials (n = 5). Student’s t-test compared to the negative control: *p < 0.05, **p < 0.01, and ***p < 0.001; comparison between the lines: #p < 0.05, ##p < 0.01, and ###p < 0.001.

Figure 2. Rab8 localization around the IBs of OPTN E478G.
Confocals microscopic observation of transiently mCherry-tagged Rab8A-expressed and immunostained Neuro2a cells expressing GFP-OPTN-WT or -E478G. (A) Confocal image of live Neuro2a cells transiently expressing GFP-OPTN-E478G (green) and mCherry-Rab8A or mCherry monomer as a control (magenta). Bars = 5 µm. (B) Fluorescence images of cells containing GFP autofluorescence and Alexa Fluor 488 signal for GFP-OPTN (green) and Alexa Fluor 594 signal for Rab8 or GFP (magenta), and their merged images (merge). White arrows indicate the IB position of the E478G mutant of OPTN. Bars = 5 µm. (C) Fluorescence images: cropped images from (B). Yellow arrows
indicate lines of interest for the intensity profile. The bottom graph indicates the fluorescence intensity plots of the position of the yellow arrows. Green and magenta lines indicate Alexa Fluor 488 with GFP and Alexa Fluor 594 fluorescence intensity, respectively. Black arrowheads show the position of the edge of the OPTN IBs.

**Figure 3. Rab8A depletion decreases IBs of OPTN E478G.**

(A) Confirmation of knockdown efficiency by western blot. Cells lysed just after the acquisition of the fluorescence images were analyzed. (top) The lysate of non-specific (NS) siRNA-transfected cells as a negative control was diluted from the undiluted lysate by 2/3, 1/2, 1/4, and 1/8, and then applied to the SDS-PAGE. (bottom) Western blot of Rab8A-knocked down cell lysate using an anti-α-tubulin antibody as a loading control. (B) Comparison of IB formation of E478G mutant of OPTN between Rab8A knocked down and control cells. Bars and dots indicate the mean, SD, and values of 3 independent trials, respectively (>240 cells per one trial were counted). Student’s $t$-test: **$p$ < 0.01.

**Figure 3. A proposed model for conformational changes and aggregation of OPTN.**

**References**


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