Lys-63-linked Ubiquitination by E3 Ubiquitin Ligase Nedd4-1 Facilitates Endosomal Sequestration of Internalized α-Synuclein*

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α-Synuclein (aS) is a major constituent of Lewy bodies, which are not only a pathological marker for Parkinson disease but also a trigger for neurodegeneration. Cumulative evidence suggests that aS spreads from cell to cell and thereby propagates neurodegeneration to neighboring cells. Recently, Nedd4-1, a neural precursor cell expressed developmentally down-regulated protein (1), has been shown to catalyze the Lys-63-linked polyubiquitination of intracellular aS and thereby facilitate aS degradation by the endolysosomal pathway. Because Nedd4-1 exerts its activity in close proximity to the inner leaflet of the plasma membrane, it is speculated that the internalization of aS in the membrane resident aS is preferentially ubiquitinated by Nedd4-1. To clarify the role of Nedd4-1 in aS internalization and endolysosomal sequestration, we generated aS mutants, including ΔPR1(1–119 and 129–140), ΔC(1–119), and ΔPR2(1–119 and 134–140), that lack the proline-rich sequence, a putative Nedd4-1 recognition site. We show that wild type aS, but not ΔPR1, ΔPR2, or ΔC aS, is modified by Nedd4-1 in vitro, acquiring a Lys-63-linked ubiquitin chain. Compared with the mutants lacking the proline-rich sequence, wild type aS is preferentially internalized and translocated to endosomes. The overexpression of Nedd4-1 increases aS in endosomes, whereas RNAi-mediated silencing of Nedd4-1 decreased endosomal aS. Although aS freely passes through plasma membranes within minutes, a pulse-chase experiment revealed that the overexpression of Nedd4-1 markedly decreased the re-secretion of internalized aS. Together, these findings demonstrate that Nedd4-1-linked Lys-63 ubiquitination specifies the fate of extrinsic and de novo synthesized aS by facilitating their targeting to endosomes.

The intraneuronal aggregation of misfolded α-synuclein (aS), known as a component of Lewy bodies (LB), is a pathological hallmark of Parkinson disease (PD). After the discovery of LB-like inclusions in the grafted neurons of PD patients who had previously received transplants of fetal mesencephalic neurons (1), increasing evidence has suggested that both monomeric and oligomeric aS can be secreted into the extracellular milieu (2), thereby affecting the physiological state of neighboring cells. Previous studies have revealed that the cellular uptake of fibrillar aS requires physiological temperatures and dynamin-1 (3, 4), a master regulator of endocytic vesicle formation, suggesting the
active participation of the endocytic machinery. However, aS incorporation into cells is not completely disabled by the inhibition of endocytosis (3), indicating that other pathways, such as direct penetration, macroendocytosis, pore formation, and diffusion, might be involved in aS internalization (5). This notion is supported by previous studies showing that both monomers and oligomers of aS can freely pass though plasma membranes (4).

Because the aggregated aS in the brains of PD patients is robustly ubiquitinated (6–8), the ubiquitination modification of aS may regulate the biogenesis of LBs and could contribute to neurodegeneration in PD. Among the E3 ligases that catalyze aS ubiquitination, researchers have focused on Nedd4 (neural precursor cell expressed developmentally down-regulated protein 4) because this E3 ligase is highly expressed in neurons containing LBs and catalyzes the Lys-63-linked ubiquitination of aS (9, 10). Mammalian Nedd4 exists in two isoforms, Nedd4-1 and Nedd4-2. Structurally, the Nedd4 isoforms are composed of a C2 domain, 3–4 WW domains, which recognize proline-rich motifs (PPXYP or LPXYY) in substrate proteins (11, 12), and a catalytic domain homologous to the E6-AP C-terminal (HECT) domain at the C terminus. Nedd4-1 binds to the plasma membrane via the C2 domain in a Ca2+ dependent manner (13) and controls the ubiquitination of membrane-bound receptor proteins, such as the epithelial Na+ channel (11). Modification by Lys-63-linked ubiquitination promotes the sorting of receptors for endocytosis and their subsequent degradation through the endolysosomal pathway (14). Given that Nedd4-1 exerts its E3 ligase activity in close proximity to the inner leaflet of the plasma membrane, the catalytic activity of Nedd4-1 likely acts preferentially on membrane-resident aS. In this study, we explored the possible mechanism by which the ubiquitination of aS by Nedd4-1 in the juxtamembrane cytoplasm could contribute to the incorporation and endosomal targeting of aS.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Plasmid Preparation, and Transfection**—SH-SY5Y human dopaminergic neuroblastoma cells (ATCC, Manassas, VA) were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Thermo Scientific, MA) at 37 °C under humidified 5% CO2/air. Triple FLAG-tagged human wild type Nedd4-1 was transfected with 5% CO2/air. Triple FLAG-tagged human wild type Nedd4-1 was transfected with pCMV10 vector. The HECT active-site dead mutant, C867A, and WW1–4-deleted (WW) mutant were produced using the PrimeSTAR mutagenesis basal kit (TaKaRa, Otsu, Japan). All recombinant proteins were expressed in the BL21(DE3)pLysS strain and purified as described previously (3). The purity and identity of the recombinant proteins were verified by Coomassie Brilliant Blue (MP Biomedicals, OH) staining and Western blot analysis. To verify the native state of recombinant aS, proteins were separated by blue native-PAGE (BN-PAGE). Briefly, the samples were charged by BN sample buffer (50 mM imidazole, pH 7.0, 50 mM NaCl, 5 mM 6-amino-hexanoic acid, 0.5% Coomassie G-250, 1.0% digitonin, 20% glycerol) and then subjected to Any-KD™ TGXTM gradient gel (Invitrogen) with cathode buffer (0.02% Coomassie G-250, 50 mM Tricine, 7.5 mM imidazole, pH 7.0) and anode buffer (25 mM imidazole, pH 7.0). The proteins were electrophoresed for 20 min at 200 V, 4 °C, followed by a change of the Coomassie G-250 concentration of the cathode buffer to 0.002%, electrophoresis was continued for 60 min at 200 V, and then electroblotted onto a PVDF membrane.

**In Vitro and in Vivo Ubiquitination Assays**—The in vitro ubiquitination assay was performed according to the manufacturer’s instructions (Enzo Life Sciences, New York). Briefly, 10 nM recombinant aS and 0.5 μg per reaction of the E3 ubiquitin ligase (E3) described below were incubated with 125 nM biotinylated ubiquitin, 5 nM E1 ubiquitin-activating enzyme (E1), 250 nM E2 ubiquitin-conjugating enzyme (E2), 250 μM Mg-ATP, and 10 units/ml inorganic pyrophosphatase (Sigma) at 37 °C for 30 min, and the reaction was quenched with 2× Laemmli buffer. All materials other than E3 and inorganic pyrophosphatase were obtained from Enzo Life Sciences. The E2s used were as follows: Ubch1, Ubch2, Ubch3, Ubch5a, Ubch5b, Ubch5g, Ubch6, Ubch7, Ubch8, Ubch10, and Ubch13/ Mms2 (Enzo Life Sciences). The E3s used were as follows: SIAH-1 (Abnova, Taipei, Taiwan), SIAH-2 (Abnova), CHIP (Millipore), Hsp70 (Enzo Life Sciences), E6-AP (Boston Biochem), Nedd4-1 (Abcam), and Nedd4-2 (Abnova).

**RNAi Interference**—To ablate Nedd4 expression in cultured cells, siRNA specifically targeting human Nedd4-1 (sc-41079, Santa Cruz Biotechnology) or Nedd4-2 (NEDD4LHSS118599, Invitrogen) or a scrambled control siRNA (sc-36869, Santa Cruz Biotechnology) was used. To silence human CHMP2B, a target-specific siRNA (sc-72895, Santa Cruz Biotechnology) was used. For human aS silencing, a 25-nucleotide-long siRNA was used, 5′-GACCCAGAGCAAGGACAAAAUGUU-3′ (BONAC, Kurume, Japan) (15). SH-SY5Y cells in low phase growth were transfected with target-specific or control-scrambled siRNAs by electroporation. Then 24 h after gene silencing, 5 μM recombinant aS was added to the culture media, and the cells were incubated for another 24 h.

**Subcellular Fractionation**—For the subcellular fractionation of cultured cells, we adopted an established protocol (16). After being cultured for 24 h in medium containing 5 μM aS, the cells (1 × 107) were resuspended in 1 ml of ice-cold buffer (10 mM Tris/acetic acid, pH 7.0, and 250 mM sucrose) and homogenized using 20 strokes in a 2-ml Dounce tissue grinder. In some experiments, the cells were pretreated with 5 μM chloroquine (CQ, Sigma) and/or 10 μM MG132 (Millipore/CaliBiochem) before exposure to aS. The cell homogenate was initially cleared by centrifugation (4000 × g for 2 min) to remove debris, destroyed cells, plasma membrane, and nuclei. The supernatant was ultracentrifuged at 100,000 × g (Hitachi Koki Co., Ltd., Tokyo, Japan) for 2 min to pellet the mitochondria, endosomes,
Western Blot Analysis—After measuring the protein concentration using a bicinchoninic acid protein assay kit (Thermo Scientific), lysates containing 20 μg of protein were electrophoresed, and the separated proteins were then electroblotted onto a PVDF membrane. After blocking with TBST containing 5% nonfat dry milk, the membranes were incubated with the following: anti-FLAG/M2 mouse monoclonal Ab (mAb) (1:1000, Sigma); anti-HA mouse mAb (1:1000, CST); anti-synuclein-1 mouse mAb (1:1000, BD Biosciences); anti-aS rabbit polyclonal Ab (pAb) (2628; 1:1000, CST); anti-aS mouse mAb (Syn211; 1:1000, Sigma); anti-SIAH-1 rabbit pAb (1:1000, Abnova); anti-SIAH-2 rabbit pAb (1:1000, Sigma); anti-CHIP rabbit pAb (1:1000, Santa Cruz Biotechnology); anti-Hsp70 (1:1000, StressGen); anti-E6AP mouse mAb (1:1000, Enzo Life Sciences); anti-Nedd4-1 rabbit pAb (1:1000, Abcam); anti-Nedd4-2 rabbit pAb (1:1000, CST); anti-BSA polyclonal Ab (1:2000, Santa Cruz Biotechnology); anti-Hsp90 mouse mAb (1:4000, StressGen); anti-ubiquitin Ab (P4D1; 1:1000, Santa Cruz Biotechnology); anti-Nedd4-1 rabbit mAb (1:500, Abcam); and anti-ubiquitin mouse mAb (1:500, Santa Cruz Biotechnology). The primary antibodies were followed by HRP-conjugated secondary Ab (1:10,000, Jackson ImmunoResearch). The bands were visualized with the Lumina™ Forte Western HRP substrate (Millipore), and the images were captured using the Omega Lum G imaging system (Aplegen, Pleasanton, CA). All experiments were performed at least three times, and each of the bands was digitalized using ImageJ software (National Institutes of Health). Differences between the conditions were analyzed with Dunnett’s multiple comparisons test using GraphPad Prism, version 6, for Mac OS X (GraphPad Software). The data are expressed as the means ± S.E.

RESULTS

aS Is Internalized and Accumulates in the Endosomes of Neuronal Cells—First, we assessed the molecular weight of recombinant aS by BN-PAGE. The total recombinant aS migrated at ~40 kDa and showed two smaller peaks over 700 kDa (Fig. 1a). To eliminate the HMW aS species, recombinant aS was further separated using a 100-kDa pore-size filter, and LMW aS migrating at ~40 kDa was collected (designated as <100-kDa aS in Fig. 1a). To visualize the internalized aS, we prepared less than 100-kDa aS covalently bound to a fluorescent compound (Alexa-aS). The labeling efficiency was estimated to be ~2.29 (mol of dye)/(mol of protein) by fluorometric analysis (Alexa-aS in Fig. 1b). In BN-PAGE, Alexa-aS has two peaks, and the conformational changes might be caused during the process of fluorescence labeling or size exclusion chromatography (Fig. 1c). After treatment with 5 μM Alexa-aS for 24 h, acceptable amounts of internalized aS were detected in SH-SY5Y cells (Fig. 1d). Subcellular fractionation analysis revealed that internalized aS appeared in the cytosol in the early phase (8 h) and thereafter gradually increased to 24 h. However, the increased aS in the endosomes and lysosomes was inversely proportional to the cytosolic aS, indicating the translocation of cytosolic aS to endo/lysosomal compartments (Fig. 1e). The kinetics of aS re-secretion into the medium was similar to that of the cytosolic aS. It should be noted that the endosomal fraction isolated by our method contains both early and late endosomes because this fraction is positive for Rab5 and Rab7 (Fig. 1e). Next, we
performed immunocytochemical analysis using SH-SY5Y cells expressing enhanced GFP-tagged Rab5a, Rab7, and Rab11a together with the acidic organelle marker LysoTracker or the mitochondrial marker MitoTracker. Most of the internalized aS co-localized with Rab7-positive late endosomes and LysoTracker-positive structures and co-localized to a lesser degree with Rab5a-positive early endosomes and Rab11a-positive recycling endosomes. Note that none of the Alexa-aS corre-
sponds with the fluorescence of MitoTracker (Fig. 1f). Another intriguing finding is that large Alexa-aS inclusions were occasionally surrounded by Rab7-positive vesicular structures. The level of internalized aS was in proportion to the amount of aS added to the culture medium (Fig. 1g). We also observed re-secreted aS in the medium 1 h after replacing it with fresh medium. The endosomal fraction used in this study also contains mitochondria, Golgi, autophagosomes, and endoplasmic reticulum because this fraction was positive for mitochondrial Tom20, the Golgi marker, syntaxin-6, the autophagosome marker p62, and the endoplasmic reticulum marker BiP (Fig. 1g). To exclude the possibility that internalized aS was located in these organelles, SH-SY5Y cells treated with Alexa-aS were fixed and subjected to double immunostaining. As shown in Fig. 1h, none of the Alexa-aS-positive red dots were co-localized with endoplasmic reticulum proteins (calnexin and PDI) or syntaxin-6. In addition, we performed p62 immunostaining using the aS-exposed, Nedd4-1-expressing cells in the presence of the autophagy inhibitor CQ (Fig. 1i). The treatment with CQ caused numerous p62-positive fluorescent puncta, indicative of autophagosomes; however, the Alexa-aS-positive puncta were scarcely co-localized with p62.

Nedd4-1 Catalyzes the Lys-63- and Lys-11-linked Polyubiquitination of aS—Several E2 ligases are known to catalyze the Lys-63-linked ubiquitination of aS, leading to its endolysosomal targeting and degradation (9, 17–20). To elucidate E3 ligase activity for aS, an in vitro ubiquitination assay was performed using the following human E3 ligases: SIAH-1, SIAH-2, Parkin, CHIP, Nedd4-1, and Nedd4-2 (Fig. 2a). In agreement with previous observations (9), we recapitulated that Nedd4-1 strongly catalyzes the ubiquitination of aS species in the presence of UbcH5b. In addition, we found that the Nedd4-2 isoform equally ubiquitinates aS. The other E3 ligases failed to form ubiquitin chains on aS under the assay conditions employed. Nedd4-1 has been shown to prefer UbcH4, UbcH5b, UbcH5c, UbcH6, and UbcH7 as E2 ubiquitin-conjugating enzymes (21). To confirm this finding, aS ubiquitination by Nedd4-1 was re-evaluated with different E2 enzymes in vitro. We confirmed that UbcH5, UbcH6, UbcH7, and UbcH8 participated in the polyubiquitylation by Nedd4-1 (Fig. 2b, lower panel). Among them, HMW aS-positive smears appeared solely in the samples containing UbcH5 and UbcH6 (Fig. 2b, upper panel). This finding is contradictory to a report by Tofarisi et al. (9), which demonstrated that UbcH7 ubiquitinates aS together with Nedd4-1. This discrepancy could be due to different experimental conditions. Ubiquitin has seven lysine residues (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63), all of which can form ubiquitin chains, resulting in various structures that alter the target protein in different ways. To determine the types of ubiquitin linkages preferentially generated by Nedd4-1, recombinant wild type aS, together with Nedd4-1, was incubated with ubiquitin with only one active lysine residue (Lys-6) or ubiquitin with only one active lysine residue (Lys-6–Lys-11), Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63). A polyubiquitination smear exclusively appeared when the samples were incubated with wild type and Lys-63 ubiquitin (Fig. 2c, lower panel). Polyubiquitinated aS species were detected in the presence of wild type, Lys-11, Lys-63, and to a lesser extent Lys-33 ubiquitin, showing that Nedd4-1 preferentially promotes the assembly of the Lys-63-linked ubiquitin chain (Fig. 2c, upper panel).

Lys-63 Linkage-specific Ubiquitination Enhances the Incorporation and Endosomal Targeting of Extracellular aS—After crossing the plasma membrane, some of the internalized aS exits the membrane, and some remains in the cytoplasm for minutes. To confirm this possibility, we performed pulse-chase experiments to determine how long internalized aS remains in the cytosolic fraction. After exposure to 5 μM recombinant aS for 24 h, the cells were extensively washed with PBS and further cultured for the indicated periods (0–60 min) in fresh medium in the absence of aS. The cytosolic fraction was isolated and subjected to Western blot analysis (Fig. 3a). After the removal of aS from the culture medium, the aS in the cytosolic fraction decreased by half within 5 min, demonstrating that the free cytosolic aS can easily cross the plasma membrane and immediately disappear from the cytosol. Based on these observations, it is tempting to speculate that specific ubiquitin modifications by Nedd4-1 might prevent aS secretion by leading to the juxtamembrane localization of aS to endosomal compartments. To confirm this possibility, we investigated whether Nedd4-1 silencing affects the re-secretion of internalized aS into the culture medium. As expected, after removing aS from the medium, Nedd4-1 silencing increased the aS re-secreted in the medium, whereas the elimination kinetics of cytosolic aS seem to be unaltered (Fig. 3, a and b). In addition to this re-secretion to the extracellular space, there are several possibilities that could explain the decrease of cytosolic aS as follows: proteolysis by the ubiquitin-proteasome system and

**FIGURE 1.** Extracellular aS is readily internalized and accumulates in the endosomes of neuronal cells. a, characterization of recombinant aS. On BN-polyacrylamide gels, a large peak around the molecular mass of 40 kDa, together with smears up to 200 kDa, are shown. Two small peaks over 700 kDa (upper panel) and <100 kDa (lower panel). The results of densitometric analyses are shown in the right panel. b, immunoblot. c, on SDS-polyacrylamide gels, denatured recombinant aS appears in its monomeric form at 18 kDa. After labeling with Alexa Fluor, a slight electrophoretic mobility shift was observed. c, Alexa-labeled aS forms two bands on a BN-polyacrylamide gel. d, internalized aS was observed in SH-SY5Y cells treated with 5 μM Alexa Fluor 488-aS for 24 h. Scale bar, 20 μm. e, after treating SH-SY5Y cells with 5 μM aS (below 100 kDa) for the indicated periods (0–24 h), internalized aS appeared in the cytosol in the early phase (8 h) and thereafter gradually decreased up to 24 h. Densitometry is shown in the right panel. The values for the amount of aS monomer in the medium, whole cell, cytosolic, endosomal, and lysosomal fractions were divided by the values of BSA, Hsp90, Hsp90, Rab7, or Lamp-2, respectively. Asterisk, p < 0.01 by Dunnett’s multiple comparisons test. f, images show live cells after treatment with 5 μM Alexa Fluor 555-aS (Alexa-aS) for 24 h in the SH-SY5Y cells. Large (>2.5 μm) Alexa-aS-positive inclusions are surrounded by EGFP-Rab7-positive structures (white arrowhead). Scale bar, 10 μm. g, after treatment with recombinant aS, the level of internalized aS is in proportion to the amount of aS added to culture medium. One hour after changing with the fresh medium, re-secreted aS appears in the medium. Densitometry is shown in the right panel. Asterisk, p < 0.1 by Dunnett’s multiple comparisons test against 0, 1, or 2.5 (μM), h, after treatment with 5 μM Alexa-aS for 24 h, SH-SY5Y cells were fixed and immunostained with organelle markers. The inset is a magnified picture of the square area. Scale bar, 10 μm.
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sequestration to autophagic degradation. To investigate this, we examined the rate of aS disappearance in the presence of CQ and proteasomal inhibitors (MG132). Of note, treatment with CQ and/or MG132 did not affect the aS turnover up to 60 min (Fig. 3, a and b). These results suggest that most of the aS that disappeared from the cytosol is not removed by lysosomal and/or proteasomal degradation. To investigate the effect of Nedd4-1 on the stability of extracellularly derived aS, we then investigated the time-dependent change of internalized aS in cells in the presence of the protein synthesis inhibitor cycloheximide. In the presence of 50 μg/ml cycloheximide, the basal amount of endogenous aS in Nedd4-1-expressing cells was significantly lower compared with control cells, but the decay kinetics of intracellular aS seem to be

FIGURE 2. Nedd4-1 catalyzes the Lys-63-linked polyubiquitination of aS. a, in vitro ubiquitination assay of aS with recombinant E3 ligases. Both human Nedd4-1 and Nedd4-2 equally catalyzed aS polyubiquitination (aS-Ubn) in the presence of UbcH5b. Other E3 ligases failed to form ubiquitin chains on aS under the same conditions. Asterisk indicates the nonspecific bands. IB, immunoblot. b, aS ubiquitination by Nedd4-1 was evaluated with various E2 enzymes in vitro. Western blotting using P4D1 antibody, which recognizes both mono- and polyubiquitin, shows an HMW smear in the samples incubated with UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, and UbcH8 (lower panel). Mono-ubiquitin (Ub) bands are weakly apparent in each lane. E2 enzymes with one or two ubiquitins were occasionally detected (double asterisk). Note that the HMW aS smear (aS-Ubn) visualized by anti-aS Ab (Syn-1) was solely detected in samples incubated with wild type, Lys-11, Lys-33, and Lys-63 ubiquitin (upper panel). Several bands at ~20–40 kDa are believed to be unspecific because these bands were also detected in the reaction with Lys-0 ubiquitin (asterisk).
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ununchanged between control and Nedd4-1-expressing cells (Fig. 3c). We observed that Nedd4-1 did not influence the turnover of de novo synthesized αS (data not shown).

Nedd4-1 Facilitates the Endosomal Targeting of αS—Intracellular αS is divided into two types based on its derivation as follows: αS internalized from the extracellular space and as de novo synthesized αS. Thus, the intracellular αS of different origins may have distinct fates during the process of intracellular vesicular transport. To determine whether Nedd4 specifies the intracellular trafficking of αS of different origins, we co-expressed wild type, C867A, or ΔWW Nedd4-1 in SH-SY5Y cells stably expressing HA-tagged αS. Following 48 h of Nedd4-1 expression, the amount of αS increased in the endosomal fraction (Fig. 4a). This finding is consistent with earlier research showing that Nedd4-1 promotes the endolysosomal sorting of αS for degradation (9). After washing off the medium, we did not detect re-secreted αS within 1 h, but a detectable amount of extracellular αS was observed up to 24 h (Fig. 4a). In an inverse correlation with endosomal αS, the level of re-secreted αS was slightly decreased in wild type Nedd4-1-overexpressing cells. To monitor the trafficking of extracellular αS in more detail, endogenous αS of SH-SY5Y cells was silenced prior to rec-αS exposure (Fig. 4, b and c). Compared with the results using HA-αS expressing cells, extracellularly derived αS tended to accumulate in the endosomal compartments (Fig. 4, a and c). Furthermore, the ectopic expression of wild type Nedd4-1 considerably increased the amount of endosomal αS in cultured cells. Wild type, C867A (asterisk, p < 0.01), extracellularly derived αS tended to accumulate in the endosomal compartments. Furthermore, the ectopic expression of wild type Nedd4-1 considerably increased the amount of endosomal αS (double asterisk, p < 0.01). In contrast, the amount of αS in the medium and the lysosomal fraction slightly, but significantly, decreased in wild type Nedd4-1-expressing cells (asterisk and double dagger, respectively, p < 0.05). The densitometric values of monomeric αS in each fraction are presented in the right panel. d, Nedd4-1 expression significantly increased the size of αS-positive inclusions surrounded by Rab7-positive endosomal structures (white arrows) in SH-SY5Y cells exposed to Alexa-αS. Scale bar, 10 μm. Asterisk, p < 0.05 by two-tailed Mann-Whitney U test (right panel). e, aS internalized from the extracellular space and as de novo synthesized HA-αS in the endosome was 20% versus the cytosolic αS (§). By contrast, the internalized recombinant αS was more extensively distributed in the endosome (50% compared with the cytosolic αS). (dagger, p < 0.01; double dagger, p < 0.05; pilcrow, p < 0.05; double pilcrow, p < 0.05; asterisk, p < 0.05 by Dunnett’s multiple comparisons test). The densitometric values of mono-αS are shown in the right panel. In these cells, the level of endosomal αS was decreased (double asterisk, p < 0.05), whereas re-secreted αS was significantly increased (asterisk, p < 0.001). The densitometric values of monomeric αS in each fraction is presented in the right panel.

FIGURE 4. Nedd4 facilitates the endosomal targeting of extracellularly derived and de novo synthesized αS. a, Nedd4-1 facilitated the endosomal targeting of de novo synthesized αS in cultured cells. Wild type, C867A (asterisk), or ΔWW Nedd4-1 was co-transfected into SH-SY5Y cells stably expressing HA-tagged wild type-αS. Following 48 h of Nedd4 expression, the amount of αS significantly increased in the endosomal fraction (dagger, p < 0.05) compared with the untransfected control (unt). Re-secreted αS levels decreased in the wild type Nedd4-1 overexpressing cells (asterisk, p < 0.01). The densitometric value of HA-αS in the medium, whole cell, cytosolic, endosomal, and lysosomal fractions were divided by the values of BSA, Hsp90, Hsp90, Rab7, and Lamp-2, respectively (right panel). b, immunoblot. c, endogenous αS level substantially declined after 48 h of αS silencing in SH-SY5Y cells. c, Nedd4-1 targeted the extracellularly derived αS to the endosomal compartment. After silencing endogenous αS, SH-SY5Y cells were further transfected with Nedd4-1 constructs for 24 h, which was followed by αS exposure (5 μM for 24 h). Compared with the results in HA-αS-expressing cells (Fig. 4a), extracellularly derived αS tended to accumulate in the endosomal compartments. Furthermore, the ectopic expression of wild type Nedd4-1 considerably increased the amount of endosomal αS (double asterisk, p < 0.01). In contrast, the amount of αS in the medium and the lysosomal fraction slightly, but significantly, decreased in wild type Nedd4-1-expressing cells (asterisk and double dagger, respectively, p < 0.05). The densitometric values of monomeric αS in each fraction are presented in the right panel. d, Nedd4-1 expression significantly increased the size of αS-positive inclusions surrounded by Rab7-positive endosomal structures (white arrows) in SH-SY5Y cells exposed to Alexa-αS. Scale bar, 10 μm. Asterisk, p < 0.05 by two-tailed Mann-Whitney U test (right panel). e, aS internalized from the extracellular space and as de novo synthesized HA-αS in the endosome was 20% versus the cytosolic αS (§). By contrast, the internalized recombinant αS was more extensively distributed in the endosome (50% compared with the cytosolic αS). (dagger, p < 0.01; double dagger, p < 0.05; pilcrow, p < 0.05; double pilcrow, p < 0.05; asterisk, p < 0.05 by Dunnett’s multiple comparisons test). The densitometric values of mono-αS are shown in the right panel. In these cells, the level of endosomal αS was decreased (double asterisk, p < 0.05), whereas re-secreted αS was significantly increased (asterisk, p < 0.001). The densitometric values of monomeric αS in each fraction is presented in the right panel.
hampered extracellularly derived aS transport from the endosome to the lysosome because Nedd4-1 expression significantly increased both the occurrence and size of aS-positive inclusions surrounded by Rab7-positive endosomal structures (Fig. 4, d and e). Theoretically, the cargo’s ubiquitin modification needs to be detached before entry into the endosomal luminal space (22). Thus, it is feasible that only 3% of Alexa-labeled recombinant aS in Rab7-positive vesicles was ubiquitinated in this cellular model (Fig. 4f). The importance of ubiquitin-dependent sorting machinery is strengthened by the fact that the silencing of CHMP2B, a component of the endosomal sorting complex required for transport (ESCRT), resulted in a marked reduction of endosomal targeting of recombinant aS (Fig. 5a). As far as the subcellular distribution is concerned, de novo synthesized HA-aS in the endosome was 20% versus the cytosolic aS. By contrast, the internalized recombinant aS was more extensively

FIGURE 5. **Nedd4-1 accumulated to both *in vitro* and *in vivo* aggregate formations.** a, silencing of CHMP2B markedly reduced the amount of recombinant aS in the endosomes (*asterisk, p < 0.01*). The densitometric values of monomeric aS in each fraction are presented in the right panel. IB, immunoblot. b, immunocytochemical analyses of aS inclusions *in vitro*. Nedd4-1-expressing SH-SY5Y cells were treated with 5 μM Alexa-aS for 24 h and then subjected to immunostaining. aS-positive dense signal over 3 μm diameter was defined as an inclusion. Note that aS-positive inclusions were partly co-localized with Nedd4-1 and CHMP2B. Scale bar, 10 μm. c–e, immunohistochemical analysis of substantia nigra from postmortem PD brain. The core structure of Lewy bodies showed immunoreactivity with Rab7A (c), and Nedd4-1 (d, Millipore; e, Sigma). Scale bar, 10 μm.
distributed in the endosome (50% compared with the cytosolic aS) (Fig. 4g). To further establish the role of Nedd4 in the endosomal targeting of aS, we knocked down endogenous Nedd4-1 or Nedd4-2 prior to aS exposure. As shown in Fig. 4h, a substantial decrease of endosomal aS and an elevation of extracellular aS were observed in Nedd4-1-deficient cells, although this effect was unremarkable in Nedd4-2-silenced cells. This finding may indicate that Nedd4-1 and Nedd4-2 are functionally distinct and that Nedd4-1 is the main human Nedd4 isoform that affects the fate of internalized aS in human neuronal cells.

Nedd4-1 Is a Component of aS-positive Inclusions in Cellular Model and the Brain Lesion of PD—To verify the functional role of Nedd4-1 in the formation of aS-positive inclusions, SH-SY5Y cells transfected with Nedd4-1 were treated with Alexa-aS for...
24 h and then subjected to double immunostaining. As shown in Fig. 5b, aS-positive inclusions were partially positive for Nedd4-1 and a component of ESCRT machinery, CHMP2B. This outcome indicates that the ESCRT pathway is closely involved in the process of aS inclusions formation. Furthermore, we found that the core structure of LBs, a pathological hallmark of PD, showed strong immunoreactivity with Rab7A (Fig. 5c) and Nedd4-1 (Fig. 5, d and e).

C-terminal Residues of aS Are Required for Nedd4-1-mediated Endosomal Targeting—Nedd4-1 recognizes PR regions of target proteins via the WW domain, thereby exerting its E3 ligase activity. Although aS does not have a canonical PPXY motif, it contains a relatively proline-rich domain (PVD/DNAYEMP128EGYQDYGEPEA) at its C terminus (9). To determine the functional importance of the PR sequence in Nedd4-1-mediated ubiquitination, three deletion mutants of aS, designated ΔPR1(1–119 and 129–140), ΔPR2(119–134 and 140–150), and ΔC(1–119), were generated and expressed in E. coli (Fig. 6, a and b). To discriminate among these mutants by Western blotting, the following Abs were used: Syn-1, Syn211, and 2628. The Syn-1 Ab, which recognizes aa 91–99, detected all mutant proteins, whereas the Syn211 Ab, which recognizes aa 121–125, did not detect all mutant proteins. The 2628 antibody, the exact specificity of which is unknown, detected wild type aS but not ΔPR2 and ΔC aS (Fig. 6b). Not only wild type aS but also mutant aS migrated to ~30–40 kDa (Fig. 6c). After the in vitro ubiquitination assay, all recombinant proteins were subjected to Western blotting using the Syn-1 Ab. We found that wild type aS produced high molecular weight bands in the presence of UbcH5b. In contrast, high molecular weight bands appeared less noticeable in samples containing these mutants (Fig. 6d). These results provide evidence that the PR sequence is required for aS ubiquitination by Nedd4-1. To further substantiate and extend these observations, SH-SY5Y cells were exposed to 5 μM wild type aS or to mutant aS lacking the PR sequence for 24 h. Intriguingly, we found that the endolysosomal aS as well as the cytosolic targeting of all mutants was greatly inhibited as compared with that of wild type aS, whereas the PR mutation substantially increased the level of intracellular aS. The level of intracellular aS was significantly decreased in P120A mutant aS, whereas P120A substitution substantially increased the amount of re-secreted aS in the medium (asterisk, p < 0.01; each mutant is compared with wild type aS, Dunnett’s multiple comparisons test). The right panel shows the amount of monomeric aS normalized by each fraction marker. IB, immunoblot.

![Figure 6](image)

**Pro-120 and Pro-128 in the PR Sequence Are Essential for Nedd4-1-mediated Endosomal Targeting of aS**—To further elucidate which proline residue(s) within the PR sequence are essential for the Nedd4-1-mediated aS ubiquitination, we generated recombinant aS in which proline 120 or proline 128 was replaced with alanine (P120A and P128A, respectively, in Fig. 7, a and b). Because HECT-type ubiquitin ligase sometimes attaches to phosphorylated serine/threonine residues (23), serine 129 (Ser-129), which is a major phosphorylation site in aS, was also substituted with alanine (S129A, in Fig. 7, a and b). After size exclusion filtration using a 100-kDa Amicon filter, all mutant aS and wild type aS migrated to ~40 kDa on BN-PAGE (Fig. 7c). In vitro ubiquitination assays using the UbcH5b E2 enzyme revealed that P120A and P128A aS mutants were less prone to being polyubiquitinated by Nedd4-1 compared with wild type aS, whereas the ubiquitination of S129A aS by Nedd4-1 was comparable with that of wild type aS (Fig. 7d), suggesting that Pro-120 and Pro-128 are key residues for the Nedd4-1-mediated ubiquitination of aS. Intriguingly, we found that the level of intracellular aS was slightly decreased in P120A mutant aS, whereas P120A substitution substantially increased the amount of re-secreted aS in the culture media (Fig. 7e). Furthermore, subcellular fractionation analysis showed that the cytosolic and endosomal targeting of P120A and P128A mutants was disturbed compared with wild type and S129A aS. Cumulatively, these findings confirm the functional importance of the Pro-120 and Pro-128 residues for Nedd4-1-mediated endosomal targeting of aS.

**DISCUSSION**

One of the most exciting themes emerging from recent neurodegenerative research is the transcellular spread of pathogenic protein aggregates in affected brain lesions. To understand how aggregated proteins, such as aS, travel from cell to cell, the underlying mechanism responsible for the uptake and secretion of aggregate-prone proteins must be elucidated. The internalization of aS by cells is thought to be initiated by aS attachment to the outer leaflet of the plasma membrane via its amphipathic N-terminal domain (24, 25), which induces membrane curvature, tubulation, and breaking (26). Prior evidence has suggested that endocytic processes play a role in aS internalization in both neuronal and glial cells (3, 4, 27); however, aS internalization was not found to be completely blocked by the disruption of the endocytic machinery. These findings indicate that mechanisms other than endocytosis may contribute to aS internalization (3, 4, 27). Indeed, there is evidence showing that fibrillar and nonfibrillar oligomeric aS species are incorporated via the endocytic machinery and that monomeric aS directly passes through the plasma membrane (4). Unfortunately, how aS crosses the plasma membrane remains to be determined.
Several possibilities have been postulated, including direct penetration (25), the formation of annular pore-like structures (28), and macropinocytosis (5, 29). Although this notion is provocative, it is supported by analogous studies in other neurodegenerative diseases, such as polyglutamine disease, in which polyQ aggregates can rapidly enter the cytosolic compartment of mammalian cells and nucleate the aggregation of soluble proteins with these polyQ tracts (30). Regardless of the mecha-
nisms involved in aS internalization, some extrinsic aS species can likely enter neuronal and/or glial cells directly, where they gain access to the cytosolic compartment and are subjected to further processing, modification, and transport.

In this study, we found that Nedd4-linked Lys-63 ubiquitination specified the fate of extrinsic and de novo synthesized aS by facilitating aS targeting to endosomal compartments. It appears that immediately after passing through the plasma membrane, the majority of the internalized aS is located just beneath the plasma membrane. Because Nedd4 localizes to the cytosolic space by associating with the inner plasma membrane leaflet via its C2 domain, Nedd4-1 likely preferentially catalyzes juxtamembrane aS localization. This notion is supported by previous studies showing that Nedd4-1-mediated ubiquitination is closely linked to the turnover and trafficking of cell-surface receptors (31, 32). Although aS does not contain the canonical PPXY motif known to interact with the WW domain of Nedd4-1, the WW domain can recognize several motifs other than PPXY with varying affinities (33). For example, the WW domains of Nedd4 families recognize PPLP, PR motifs, and phospho-(Ser/Thr) residues, as well as PPXY (23). Our observation using deletion mutants provides evidence that the PR sequences in the C terminus of aS, particularly residues Pro-120 and Pro-128, are important for proper recognition by Nedd4-1. It is uncertain why Nedd4 silencing did not show a similar effect on the cytosolic aS accumulation as the H9004 PR and P120A mutations. However, this could be attributed to the insufficient silencing efficacy of Nedd4 in cultured cells. The fact that a major phosphorylation site, Ser-129, occurs in the region flanking the aS PR sequence (34, 35) raises the possibility that phosphorylation at Ser-129 might affect Nedd4-1-mediated ubiquitination. However, this effect is not likely, as we found no difference in aS ubiquitination regardless of the presence of Ser-129.

Nedd4-1 catalyzes both the mono-ubiquitination and Lys-63-linked polyubiquitination of target proteins (36). Lys-63-linked polyubiquitination is implicated in various cellular activities, including protein trafficking, DNA repair, stress responses, and signal transduction (37). Although mono-ubiquitination appears to be involved in endocytic trafficking, additional Lys-63-linked polyubiquitination is known to accelerate this trafficking process (38). More specifically, Lys-63-linked polyubiqui-
utination serves as a signal for protein sorting into the ESCRT-driven multivesicular body pathway by inward membrane invagination of endosomes (39). Previous studies have shown that LB showed immunoreactivity against ESCRT components such as CHMP2B and VPS4 (2, 40, 41). These findings are interesting when considering the biogenesis of LB because the pale body, a possible precursor of LB, often contains lysosomes, vacuolar structures, and ubiquitinated proteins (42). Moreover, our observation that Nedd4-1 is a component of LB also strengthens the hypothesis that the Nedd4-regulated endo/lysosomal sorting machinery might be involved in the buildup of aS-positive aggregates in affected brain lesions.

The mechanism by which cytosolic aS moves into the endosomal vesicle is poorly understood; however, our result showing that the silencing of CHMP2B, a component of ESCRT-III, can disrupt the endosomal accumulation of aS indicates the functional relevance of ESCRT machinery in the endolysosomal targeting of aS. Mechanistically, the endosomal targeting of ubiquitinated cargo and the formation of multivesicular bodies are mediated by the upstream ESCRT complexes (ESCRT-0, -I, and -II) on the surface of the endosomal membrane. ESCRT-III then recruits de-ubiquitinating enzymes to remove ubiquitin from the cargo before incorporating them into the intraluminal vesicles of multivesicular bodies (43, 44). This could be a reason why we failed to detect strong ubiquitination in aS-positive inclusions surrounded by Rab7-positive late endosomes. Another important finding of this study is that the Nedd4-1-mediated endosomal targeting of aS was accompanied by a prominent enlargement of the late endosome. Intriguingly, a marked enlargement of endosomal vesicles has also been shown in the affected brain and in a cellular model of Alzheimer disease (45, 46). Why the overexpression of Nedd4-1 down-regulated the lysosomal accumulation of aS in this study is uncertain. One possible explanation is that the excessive accumulation of aS might prevent early-to-late endosome transition. Indeed, previous studies have shown that aS itself is closely involved in vesicular trafficking events, such as Rab-mediated endoplasmic reticulum-Golgi transport and endosomal trafficking (47–49). An alternative possibility is that aS aggregates may decrease the lysosomal burden by inducing lysosomal rupture (50).

In summary, we found that Nedd4-1 markedly facilitated aS internalization, which was linked to Lys-63 linkage-specific polyubiquitination. Our results demonstrate how Lys-63-linked ubiquitination contributes to the endosomal targeting and the endosomal accumulation of aS and therefore may be involved in the propagation and formation/clearance of Lewy pathology in PD (Fig. 8). Although the concept of the cell-to-cell transmission of aberrant proteins has been recognized as a common phenomenon in many neurodegenerative diseases, the molecular mechanisms underlying the spread of protein misfolding likely differ depending on the biochemical nature of the protein aggregate, the level of cellular stress, and the cell type. Further studies are needed to gain insight into the cellular mechanisms of disease progression and to identify molecular targets for therapeutic intervention in PD and other neurodegenerative diseases.

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