Lysosomal enzyme cathepsin B enhances the aggregate forming activity of exogenous α-synuclein fibrils

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α-Synuclein (α-Syn) is a major component of Lewy bodies (LBs), which are the intracellular pathological inclusions seen in synucleinopathies such as Parkinson’s disease (PD), and dementia with Lewy bodies (DLB). α-Syn oligomers/fibrils are considered to underlie the disturbances of a number of intracellular processes including mitochondrial functions (Proter et al., 2012) and protein degradation processes (Xilouri et al., 2013), which may finally induce neuronal death. Studies using the brains of patients with PD have indicated that LB and Lewy neurite pathologies occur first in a few specific regions of the lower brain stem and olfactory bulb, and then spread into other regions of the brain as the disease progresses (Braak et al., 2004). Furthermore, recent studies have demonstrated the ability of α-Syn fibrils to transfer between cells, both in cultured cells and in mice (Desplats et al., 2009; Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011). Thus, it is now considered that the propagation of LB pathology in the brains of PD patients is in part due to the transmission of pathogenic polymerized forms of α-Syn. Knowledge about each step of this α-Syn transfer is being accumulated; for example, it was found that pathologica...
the lysosome, enhances the aggregate forming activity of α-Syn fibrils, and that intracellular α-Syn aggregates begin to appear in the lysosome.

Materials and methods

Chemicals and antibodies

Three α-Syn antibodies, anti-α-Syn polyclonal antibody (C20) {Santa Cruz Biotechnology, Inc., CA}), anti-α-Syn monoclonal antibody (syn-1) {BD Bio Sciences, New Jersey} and anti-phosphorylated α-Syn (pSyr) antibody (Ser129) {Wako Pure Chemical Industries Ltd, Osaka, Japan} were purchased in this study. Anti-GFP and anti-LC3 antibodies were purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Anti-LAMP-2 monoclonal antibody was purchased from Bior Cancer Science (Bristol, UK). Protease inhibitors, pepstatin A, CA-74Me, and E-64d were purchased from Peptide Institute Inc. (Osaka, Japan), and LysoTracker Red DND-99 was purchased from Thermo Fischer Scientific Life Technologies Invitrogen (Massachusetts).

Cell culture

Cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Human α-Syn cDNA was cloned into pEGFP-N1 (TAKARA BIO INC., Otsu, Japan) and the resulting expression vector was transfected into HEK293F cells with Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen). After selection with 400 μg/ml G418, a clonal-derived HEK293F/Syn-ECFP cell line was used in the intracellular aggregate-forming assay.

Preparation of recombinant α-Syn and its aggregates

Human α-Syn cDNA was amplified by PCR (forward primer, 5′-ATGATGATATATGAACTGACAAAGT-3′; reverse primer, 5′-AGGCTCATAGTC-3′) and the resultant fragment was digested with NdeI and HindII, and then cloned into NdeI-HindIII sites of the pET43.1a E. coli expression vector (Merck Millipore, MA). The PrimeSTAR Mutagenesis Basal Kit [Takara Bio Inc.] was used to make a α-Syn S129A point mutation according to the manufacturer’s instructions using the synthetic primers S129A_FW 5′-CTGTCTGACAGAAGGATATCAAGCT-3′ and Mut-Rel 5′-CATTCTAAGCCCTAAGTCAGATC-3′. Recombinant α-Syn was expressed in E. coli strain BL21 codon plus (DE3) (Agilent Technologies, Santa Clara, CA) with 1 mM IPTG induction and recovered by osmotic shock into buffer consisting of 25 mM Tris–HCl/1 mM EDTA, pH 8.0. The recovered periplasmic fraction was boiled for 10 min and insoluble debris was removed by centrifugation. The cleared supernatant containing recombinant α-Syn was charged on a Hitrap Q column (GE Healthcare, UK) and eluted with a 0–1 M NaCl linear gradient. Finally, the protein was desalted with disposable PD-10 Desalting Columns with PBS and stored at −20 °C.

Fibrill forms of α-Syn were prepared in accordance with a previous report {Volpicelli-Daley et al., 2011}. Briefly, fibrils of α-Syn were generated by incubating purified α-Syn in PBS at a final concentration of 2 mg/ml with constant agitation (500 rpm) at 37 °C for 168 h. Then, fibrils were recovered by ultracentrifugation (110,000 × g, 20 min), resuspended in PBS with brief sonication and stored at −20 °C. In vitro protein aggregation was monitored using the ProteoStat protein aggregation assay kit (Enzo Life Sciences, Plymouth Meeting, PA) and a CytoFluor2300 system (Thermo Fisher Scientific, Applied Biosystems) equipped with excitation 530 nm and emission 590 nm filters.

Intracellular aggregate forming assay

For the assay, 1 μg or less of α-Syn monomer/oligomer/fibrils in 25 μl of DMEM medium (resuspended with brief sonication) and 1 μl of DMRIE-C transfection reagents (Thermo Fisher Scientific, Invitrogen) in 25 μl DMEM were combined and incubated for 20 min at room temperature. The complex solution was then mixed with 50 μl of trypsin-isolated HEK293F/Syn-ECFP cell suspension (1 × 10^5/50 μl) in 96-well plates. After incubation for 30 min at 37 °C, 100 μl of DMEM medium containing 20% FBS was added and incubated for a further 16–48 h in a 5% CO2 incubator. The formation of aggregates in the transfected cells was monitored using a fluorescence microscope. For the quantitation of intracellular aggregation, the culture medium was discarded by decantation and the cells were overlaid with PBS containing 0.1% Triton X-100 and 0.1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) followed by gentle rotation for 30 min in the dark. The Triton X-100-insoluble punctate fluorescence images were photographed using a IX71 fluorescence microscope (Olympus, Tokyo, Japan) and analyzed using the image-processing program “Image J” with “Analyze Particle” menu {Schneider et al., 2012}.

For inhibitor assay, cells were treated for 30 min in DMEM medium with respective compound before addition of α-Syn fibrils. After 30 min incubation with fibrils, 100 μl of DMEM medium containing respective inhibitor and 20% FBS was added.

The effect of in vitro digestion of α-Syn fibrils with cathepsin B

In vitro-prepared α-Syn fibrils (500 μg/ml) were digested with proteinase K (concentration range 48–3125 ng/ml in PBS) or cathepsin B (concentration range 16–1000 ng/ml in 25 mM MES pH 5.0/1 mM EDTA/1 mM DTT) at 37 °C for 60 min followed by heat inactivation of enzymes at 95 °C for 5 min. Digested fibrils were analyzed by Western blotting of intracellular aggregate formation and Western blotting.

Knockdown of cathepsin expression by siRNA

siRNA sequences targeting human CTSB were synthesized by Bex Co., Ltd (Tokyo, Japan). The siRNA sequences were: sense, 5′-GAGUUA UGUUUUACCAGGAAATT-3′; anti-sense, 5′-UCCUGCUAAACAUACUCCT-3′ {Paterno et al., 2008}.

The HEK293F/Syn-ECFP cells were transfected with siRNAs at a final concentration of 25 nM using RNAiMAX (Thermo Fisher Scientific, Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were collected in trypsin for the intracellular aggregate forming assay. The efficiency of cathepsin gene knockdown was evaluated by assessing the enzyme activities of the cell lysate with the fluorogenic substrates Z-Arg-Arg-MCA and MOCA Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Leu-Lys(Dnp)-D-Arg-NH2, (Peptide Institute Inc.) according to the recommended procedure.

Knockdown of autophagy pathways by siRNAs

siRNA sequences against human ATG5 and LAMP-2 genes have been described previously {Watanabe and Tanaka, 2011; Gonzalez-Polo et al., 2005}. ATG5 gene knockdown was evaluated by suppression of LC3-II accumulation by 1 μM rapamycin (Wako Pure Chemical Industries Ltd) treatment for 6 h in the growth medium. LAMP-2 gene knockdown was by reduction of LAMP-2 protein content in the cell lysate by western blotting with anti-LAMP-2 antibody. We also confirmed reduction of both genes by quantitative PCR with appropriate primer sets: ATG5: 5′-TTGCAATGATACGCTGACAAAGT-3′/5′-TGGCAATGATACGCTGACAAAGT-3′; LAMP-2: 5′-GGCAATGATACGCTGACAAAGT-3′/5′-TGGCAATGATACGCTGACAAAGT-3′.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde (PFA) in cultured medium for 10 min at room temperature. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 5% normal goat serum (NGS) in PBS for 30 min. Next, cells were incubated with
primary antibody in blocking solution for 1–2 h. Then, the cells were washed with PBS, and further treated with secondary antibody. For double-staining, this staining procedure was repeated for each antigen and the primary antibodies were detected by Alexa488- and Alexa594-conjugated antibodies (Thermo Fisher Scientific, Molecular Probes). These cells were washed with PBS followed by DAPI staining. They were then washed with milliQ water (Merck Millipore), and finally mounted with FluorSave (Merck Millipore). Images were acquired as a Z stack (20–30 z-sections, 0.3–0.5 μm apart, 1024 × 1024) through Plan-Apochromat 40×/1.30 or 63×/1.40 Oil DIC objective lenses (Carl Zeiss, Oberkochen, Germany) with an inverted laser-scanning confocal microscope, LSM510 (Carl Zeiss) (Watanabe and Tanaka, 2011).

Time-lapse imaging of aggregate formation

For time-lapse monitoring of the intracellular aggregation formation process, HEK293F/Syn-ECFP cells were transfected with α-Syn fibrils in collagen-coated 16-well chamber slides (Thermo Fisher Scientific, Nunc, #178599) and visualized under a fluorescent microscope IX71 (Olympus) equipped with a Stage Top incubator (TOKAI HIT, Shizuoka Japan). Images were captured using a 40× objective at 15-minute intervals.

Statistics

Data are expressed as the means ± standard errors. p values were calculated by one-way ANOVA followed by Tukey’s post-hoc test.

Results

Establishment of an assay system to measure intracellular aggregate formation by α-Syn fibrils in cultured cells

To investigate the process underlying formation of intracellular LB-like aggregates, we established a HEK293F cell line stably expressing α-Syn fused to ECFP (HEK293F/Syn-ECFP cells). Under normal growth conditions, ECFP fluorescence was present uniformly in the cells and no aggregation was observed at all. When in vitro-prepared α-Syn fibrils (Luk et al., 2009) were introduced into the cells, a large condensed fluorescent signal began to appear 4 h after introduction. It was possible to observe in real-time the formation of aggregates in these cells. However, to quantify the accumulated fluorescent signals accurately was impossible because of fluorescence owing to non-participating α-Syn-ECFP in the cytoplasm. To overcome this limitation of quantification, we employed a simple washout process using 0.1% Triton X-100 (Sacino et al., 2013). Permeabilization of cell membranes allowed soluble α-Syn-ECFP to diffuse out of cells, but the detergent-resistant aggregates were retained (Fig. 1A). Varying amounts of in vitro-prepared α-Syn fibrils produced detergent-resistant fluorescent signals in HEK293F/Syn-ECFP cells, and a good correlation was obtained between the amounts of input α-Syn fibrils (Fig. 1B) and the number of particles (Fig. 1C), the total area (Fig. 1D), and the size of the signals (Fig. 1E). The insoluble α-Syn-ECFP aggregates were analyzed by conventional western blotting after separation of Triton X-100-insoluble protein by centrifugation (Supplementary Fig. S1). The levels of insoluble α-Syn-ECFP aggregates on the PVDF membranes appeared to increase in a dose-dependent manner with the amount of introduced α-Syn fibrils. However, it was considered that accurate quantification of aggregates on western blotting membranes was difficult because of the increase in the amounts of ladders and smeared bands derived from α-Syn-ECFP with a structure that is not separable on SDS-PAGE (Supplementary Fig. S1).

Monomeric α-Syn was polymerized by stirring in vitro to obtain fibril samples (Volpicelli-Daley et al., 2011), so that the process of aggregation could be monitored indirectly by an assay with fluorescent dye binding (Supplementary Fig. S2A) or sedimentation of the aggregates by ultracentrifugation (Supplementary Fig. S2B). When α-Syn monomer or in vitro samples have received ~48 h of agitation were introduced into HEK293F/Syn-ECFP cells, no intracellular aggregate formation was observed (Supplementary Fig. S2C). However, when

![Fig. 1. Quantitative seeding activity assay for α-Syn aggregates. (A) Representative images of the intracellular aggregate quantification process are shown. After introduction of α-Syn fibrils into the Syn-ECFP-expressing cells (HEK293F/Syn-ECFP cells), cells started to form aggregates in the cytoplasm within a few hours (lower left panel). After washout of free Syn-ECFP with Triton X-100, only detergent-resistant α-Syn aggregates were retained in the cytoplasm (lower middle panel). DAPI staining was performed to confirm the presence of cells and to allow correction for cell number (right panels). (B) Aggregate formation was examined using varying amounts of α-Syn fibrils. (C–E) Then, fluorescent signals were analyzed using "Image J" and presented as graphs (*p < 0.05, **p < 0.01 from the no fibril control).](image-url)
samples with a >96-h agitation period, which contained abundant fibrils (Supplementary Fig. S2B), were introduced into cells, intracellular α-Syn-ECFP aggregates were observed as bright fluorescent signals and the number and size of aggregates increased with agitation time. We decided to use fibrils created by 168 h of stirring as seeds. Measuring the total area of aggregates is considered as an appropriate means of representing the seeding activity of fibril-containing samples. The introduction α-Syn fibrils and the fibril-induced formation of intracellular aggregates for 24 h were not toxic to the HEK293F/Syn-ECFP cells based on a lactate dehydrogenase (LDH) assay, excluding a possible influence of cell toxicity from this assay (Supplementary Fig. S2D).

**Inhibition of lysosomal function suppresses α-Syn aggregate formation**

To investigate the pathways mediating intracellular aggregate formation by α-Syn, we examined several inhibitors of the proteasome and Aβ because disturbances of these degradation pathways are involved in protein aggregation (Ebrahimi-Fakhari et al., 2012). As shown in Figs. 2A and B, lactacystin, a proteasome inhibitor, had no effect on aggregate formation, but bafilomycin A1 and NH₄Cl, which neutralize lysosomal pH, strongly suppressed it in HEK293F/Syn-ECFP cells. This result suggested that lysosomal functions were involved in aggregate formation. Then, the effects of inhibitors of lysosomal enzymes on the degree of aggregate formation were examined. E-64d, an inhibitor of cysteine proteases, and CA-074Me, a cathepsin B-specific inhibitor, significantly blocked aggregate formation. Cathepsin B is a cysteine protease found abundantly in lysosomes. In contrast, pepstatin A, an inhibitor of aspartic proteases represented by cathepsin B, with proteinase K digestion used as a control. Proteinase K preferentially digests proteins after hydrophobic amino acids; therefore, proteinase K induced almost complete digestion of α-Syn fibrils and intracellular aggregates were not formed (Figs. 3A and B, left). In contrast, cathepsin B digestion enhanced intracellular aggregate formation activity, which increased together with an increase in cathepsin B concentration (Figs. 3A and B, right). Cathepsin B preferentially cleaves -Arg-Arg-|-Xaa bonds in small molecule substrates (UniProt: P07858). However, human α-Syn does not have such sequences for cleavage. Thus, the levels of partially cathepsin B-digested fibrils and slightly truncated α-Syn were present at increased levels on SDS-PAGE gels (Fig. 3B, right, arrows a and b). Moreover the band indicated by arrow ‘b’ and shorter truncated bands were not recognized by the C20 antibody, which was raised against the C-terminal region of human α-Syn (Fig. 3D). This increase in aggregate-forming activity might be attributed to C-terminally truncated fibril seeds formed owing to the dipeptidyl carboxypeptidase activity of cathepsin B with broad substrate specificity (Koga et al., 1991).

**Knockdown of the cathepsin B gene leads to decreased α-Syn aggregate formation**

To examine the effect of cathepsin B gene (CTSB) suppression on α-Syn aggregate formation, the CTSB gene was knocked down by siRNA. As shown in Fig. 4A, the enzymatic activity of cathepsin B in the cell lysate was suppressed to 45% by the CTSB siRNA. Using cells with reduced cathepsin B activity, intracellular α-Syn aggregate formation using sertraline, an endocytosis inhibitor, to suppress dynamic GTPase activity (Takahashi et al., 2010). With sertraline, we observed marked inhibition of α-Syn aggregate formation (Fig. 2C), suggesting that exogenous α-Syn fibrils enter the cell and reach lysosomes via endocytosis.

**In vitro digestion of α-Syn fibrils by cathepsin B enhances seeding activity**

As shown in Fig. 2, cathepsin B may promote aggregate formation activity. Therefore, next, we examined whether the seeding activity was influenced by the extent of digestion of α-Syn fibrils by cathepsin B, with proteinase K digestion used as a control. Proteinase K preferentially digests proteins after hydrophobic amino acids; therefore, proteinase K induced almost complete digestion of α-Syn fibrils and intracellular aggregates were not formed (Figs. 3A and B, left). In contrast, cathepsin B digestion enhanced intracellular aggregate formation activity, which increased together with an increase in cathepsin B concentration (Figs. 3A and B, right). Cathepsin B preferentially cleaves -Arg-Arg-|-Xaa bonds in small molecule substrates (UniProt: P07858). However, human α-Syn does not have such sequences for cleavage. Thus, the levels of partially cathepsin B-digested fibrils and slightly truncated α-Syn were present at increased levels on SDS-PAGE gels (Fig. 3B, right, arrows a and b). Moreover the band indicated by arrow ‘b’ and shorter truncated bands were not recognized by the C20 antibody, which was raised against the C-terminal region of human α-Syn (Fig. 3D). This increase in aggregate-forming activity might be attributed to C-terminally truncated fibril seeds formed owing to the dipeptidyl carboxypeptidase activity of cathepsin B with broad substrate specificity (Koga et al., 1991).

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was examined. About 40% suppression of seeding activity was observed in CTSB knocked down cells (Fig. 4B). Because cathepsin B was reported to be required for the activation of cathepsin D in lysosomes (Laurent-Matha et al., 2006), there was a possibility that the reduction of aggregate formation by CTSB knockdown was a result of the reduction in cathepsin D activity. However, as shown in Fig. 4A, the cathepsin D activity was not affected by CTSB knockdown, excluding the involvement of cathepsin D. Considering together the results of these experiments using specific inhibitors and siRNA, cathepsin B is considered to be responsible for the enhancement of α-Syn aggregate-forming activity. Autophagy pathways are involved in α-Syn aggregate formation

In addition to endosome trafficking, autophagy pathways are known to be involved in intracellular protein transport to lysosomes (Saftig and Klumperman, 2009). Thus, we examined the contribution of autophagy by knockdown experiments using siRNA-ATG5 or siRNA-LAMP-2. Inhibition of macroautophagy by siRNA-ATG5 treatment led to a decrease in intracellular aggregate formation to 51% of control siRNA levels (Figs. 5A–C). Inhibition of chaperone-mediated autophagy (CMA) by siRNA-LAMP-2 also decreased aggregate formation to 61% of control levels, which was comparable to reductions in LAMP-2 gene and protein expression (Figs. 5D–F). These results indicate that both macroautophagy and CMA are involved in aggregate formation after incorporation of α-Syn fibrils.

Intracellular α-Syn aggregate formation begins in the lysosome

Because cathepsin B was involved in the aggregate formation by exogenous α-Syn fibrils in the above experiments, we next examined visually the location and growth of intracellular aggregates using the anti-pSyn antibody, which reportedly recognizes phosphorylated α-Syn at position serine 129 in synucleinopathy lesions (Fujiwara et al., 2002). As shown in Fig. 6A, exogenous α-Syn fibrils were surrounded by the lysosomal membrane marker LAMP-1 at 4 h after introduction of fibrils into the cells. It was considered that the seeding activity of the introduced fibrils was acquired in the lysosomes. Then, we examined the expression of newly formed aggregates after mutant α-Syn S129A fibril introduction. This time the anti-pSyn exclusively recognizes endogenous α-Syn. Intracellular aggregates appeared with small and weak phosphorylation signals within a few hours, and then gradually increased in number and size over time (Figs. 6B–D). Regarding the changes in size, the size-distributions of aggregates on confocal

![Graph](image-url)
Microscope images are presented in a histogram (Supplementary Fig. S3). Three hours after fibril introduction, approximately 70% of the intracellular aggregates were classified into the smallest fraction. As incubation time increased, the proportion of the smallest size fraction decreased, and the amount of larger aggregates increased (Supplementary Fig. S3). It was concluded that the intracellular aggregates begin as small aggregates, and grow larger within a day in this system.

Next, we examined the growth and localization of intracellular pSyn-positive aggregates with special reference to the coexistence with LAMP-1 (Fig. 6E). Intracellular aggregates showed one of three different forms: (a) they were completely included in the LAMP-1 signals; (b) they were partially surrounded by LAMP-1 signal; or (c) they existed independently from LAMP-1 signal. For example, compared with the aggregates shown in panel (a) of Fig. 6E, larger

**Fig. 4.** Knockdown of the cathepsin B gene led to decreased aggregate-forming activity. (A) HEK293F/Syn-ECFP cells were transfected with an siRNA against the cathepsin B gene (CTSB) or a control siRNA for 2 days and cathepsin B and cathepsin D activities in the cell lysate were measured using fluorogenic substrates. The enzymatic activity of cathepsin B was decreased to 45% of the control level by the specific siRNA. The enzymatic activity of cathepsin D was not influenced by treatment with CTSB siRNA. (B) The intracellular seeding activity for aggregates formation was also measured after α-Syn fibril introduction. Similarly, seeding activity was also reduced to 56% of the control level after treatment with CTSB siRNA. Data are presented as the mean ± standard errors (**p < 0.01 from the control siRNA).**

**Fig. 5.** Involvement of autophagy pathways in aggregate formation. Autophagy pathway component genes were knocked down using specific siRNAs. (A) Images of Triton X-100 resistant α-Syn aggregates and calculated total area of intracellular aggregates in siRNA treated cells were shown. ATG5 gene knockdown reduced aggregate formation to 51% of control levels. (B) Introduction of siATG5 suppressed rapamycin induced LC3-II accumulation analyzed by Western blotting with anti LC3 antibody. (C) ATG5 gene knockdown was confirmed by quantitative PCR. (D) LAMP-2 gene knockdown reduced fibril-induced aggregate formation to 61% of control levels. (E) Intracellular content of LAMP-2 protein in siRNA treated cells was decreased to 60%, as determined by western blotting. (F) Knockdown of LAMP-2 gene expression was determined by quantitative PCR. (**p < 0.01 vs. siControl.)
aggregates with irregular shapes were seen protruding from the lysosomal structure (panel b)). Aggregates that coexisted with LAMP-1 (Figs. 6E-a and b) were counted at each point examined after fibril introduction (Fig. 6F). The percentage of coexistence of pSyn aggregates and LAMP-1 showed a peak at 6 h, and then began decreasing. At 21 h, almost no aggregates coexisting with LAMP-1 were seen (Fig. 6F).

Live-cell imaging of growing α-Syn aggregates in cells

We also performed time-lapse imaging of intracellular aggregates forming in living HEK293F/Syn-EGFP cells (Supplementary Movies S1–3). In α-Syn-EGFP expressing cells, there were always dark regions lacking EGFP fluorescence in the cytoplasm (Figs. 7A, B). After 5 h of α-Syn fibril introduction, small aggregates that appeared brighter than the cytoplasm began to appear in the dark compartments. These gradually grew larger and filled the dark places with aggregates (Figs. 7A a–c, Supplementary Movies S1–3). The cells in which aggregations grew to a large size tended to rapidly consume endogenous Syn-EGFP molecules for aggregate formation resulting in a reduction in cytoplasmic fluorescence (Figs. 7Ac). Next, we stained these dark compartments with LysoTracker or LAMP-1 antibody (at 6 h after α-Syn fibril introduction). While cytoplasmic dark compartments in HEK293F/Syn-EGFP cells were not stained with LysoTracker (white arrow heads in Fig. 7B upper panels), most of them were LAMP-1-positive (white arrows in Fig. 7B lower panels). The LysoTracker probe is used to detect acidic organelles in living cells and normal lysosomes are usually positive for both LysoTracker and LAMP-1 (Supplementary Fig. S4). These LAMP-1-positive/LysoTracker-negative dark compartments are considered to be impaired lysosomes. Based on these observations, it is strongly suggested that the initiation of aggregate formation takes place in the lysosomes after α-Syn fibril treatment, and the growth of aggregates is followed by their escape from the impaired lysosomes.

Discussion

Although several groups have reported the formation of LB-like aggregates in cells by exogenous α-Syn fibrils, how they grow into larger molecules involving endogenous α-Syn is still unknown (Luk et al., 2009; Nonaka et al., 2010; Watanabe et al., 2012; Tanik et al., 2013). To address this issue, we developed an assay system for forming detergent-insoluble α-Syn aggregates using HEK293 cells stably expressing α-Syn-ECFP. This system easily enabled the quantification of the fluorescence intensity of α-Syn-containing intracellular aggregates and quantification of the number and size of aggregates. Recently Guo et al reported that there are at least two conformational variations of sarkosyl-insoluble α-Syn in PD brains (Guo et al., 2013). Thus, in addition to assessing in vitro-prepared fibrils, the present assay system could be useful for examining pathological features in synucleinopathy patients and classifying the precise type of α-Syn pathology.

We previously observed that intracellular α-Syn inclusions, formation of which was induced by exogenous α-Syn fibrils, underwent degradation via p62/SQSTM1-dependent autophagy in HEK293 cells (Watanabe et al., 2012). Therefore, at first, we thought that inhibition of the degradation pathway would be involved in the formation of aggregates, and we treated cells with inhibitors of the ALP or the ubiquitin–proteasome system. Unexpectedly, treatment with bafloymycin A1, which blocks vacuolar H+-ATPase (V-ATPase) and prevents fusion between autophagosomes and mature lysosomes (Yamamoto et al., 1998), decreased aggregate formation, while the proteasome inhibitor lactacystin did not affect aggregate formation. Klucken et al also reported this bafloymycin A1-induced inhibition of aggregation using α-Syn-transfected H4 cells, which were prone to development of α-Syn aggregates (Klucken et al., 2012). They also showed that another V-ATPase inhibitor, chloroquine, substantially reduced aggregation, but that 3-methyladenine, which inhibits autophagy by blocking autophagosome formation, did not. Moreover, NH4Cl treatment, which blocks acidification, reduced aggregate formation in this study. Recently, Buell et al reported that, in the presence of preformed fibrils, the multiplication of aggregates was much faster at lower pH values below 6 than at normal physiological pH values in vitro (Buell et al., 2014). These lines of evidence suggest that lysosomal function is important for initiating aggregation from the seed of α-Syn fibrils.

It is generally thought that lysosomal impairment causes α-Syn accumulation (Dehay et al., 2013). In fact, concerning another abundant lysosomal enzyme, cathepsin D, there were reports that a deficiency of cathepsin D or overexpression of an inactive mutant cathepsin D caused
accumulation of α-Syn and toxicity (Qiao et al., 2008; Cullen et al., 2009; Crabtree et al., 2014). In the present study, we observed that cathepsin D inhibitor had no effect on aggregate formation. There may not have been enough time to enhance endogenous α-Syn expression and aggregate formation in this assay system. Thus, our present study revealed that, among lysosomal enzymes, cathepsin B is responsible for the activation of α-Syn seeds. This may be attributed to the fact that α-Syn does not have a typical consensus recognition sequence for cathepsin B. α-Syn fibrils pre-treated with cathepsin B in vitro were not completely digested and slightly truncated forms appeared after digestion in a high enzyme concentration on western blots. This pre-treatment with high doses of cathepsin B caused more aggregate formation in cells (Fig. 2). In the case of C-terminal truncated α-Syn, it enhances the aggregation of the more abundant full-length α-Syn in vitro and in vivo (Murray et al., 2003; Li et al., 2005; Ulusoy et al., 2010). In fact, LB extracts contain up to 15% C-terminal truncated α-Syn (Baba et al., 1998). Transgenic mice with truncated human α-Syn (1-120), driven by the tyrosine hydroxylase promoter, show pathological inclusions in the substantia nigra and olfactory bulb (Tofaris et al., 2006). Furthermore, Games et al. recently reported that reducing C-terminal truncated α-Syn by passive immunization in mThy1-α-Syn transgenic mice, attenuated neurodegeneration (Games et al., 2014).
These reports suggest that C-terminal truncated α-Syn plays an important role in pathogenesis of synucleinopathies. In this study, cathepsin B-induced partially cleaved and/or conformationally changed α-Syn fibrils are supposed to gain nucleation activity and induce formation of intracellular aggregates of α-Syn. The precise mechanism underlying the modulation of α-Syn fibrils by cathepsin B will be clarified in a future study.

Endocytosis is involved in cellular uptake of exogenous α-Syn fibrils. Our findings suggest that after endocytosis, macroautophagy plays a part in transport of α-Syn fibrils to lysosomes. We previously showed that exogenous fibrils colocalized with LC3 1 h after introduction into HEK 293 cells. As there is no endogenous α-Syn, exogenous fibrils underwent further degradation through the ALP (Watanabe et al., 2012). In the present study, suppression of α-Syn aggregate formation by siRNA-ATG5 may be owing to inhibition of α-Syn fibril trafficking before the fibrils attain seeding activity in lysosomes, as ATG5 is involved in elongation of isolated membranes in autophagosome formation (Kuma et al., 2004). On the other hand, CMA reportedly contributes to α-Syn monomer transfer to lysosomes and subsequent degradation (Cuervo et al., 2004; Xilouri et al., 2009). siRNA-LAMP-2 treatment may inhibit transport of endogenous α-Syn monomers into lysosomes and prevent initiation of intracellular aggregate formation. Further investigation will clarify intracellular transport of α-Syn fibrils after transfection.

We observed that aggregate formation begins in the lysosome. From 4 h after fibril introduction, small aggregates including endogenous phosphorylated α-Syn appeared. At first, they were surrounded by a lysosomal marker, LAMP-1 (Figs. 6E–a). Endogenous soluble α-Syn is recruited to exogenous α-Syn fibrils and is converted into insoluble, hyperphosphorylated, and ubiquitinated pathological species in the cytoplasm. Further investigation will clarify intracellular transport of α-Syn fibrils after transfection.

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