MOLECULAR BASIS OF CELL AND DEVELOPMENTAL BIOLOGY:

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Induction of Neuronal Cell Death by Rab5A-dependent Endocytosis of α-Synuclein*

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The presynaptic α-synuclein is a prime suspect for contributing to Lewy pathology and clinical aspects of diseases, including Parkinson's disease, dementia with Lewy bodies, and a Lewy body variant of Alzheimer's disease. Here we examined the pathogenic mechanism of neuronal cell death induced by α-synuclein. The exogenous addition of α-synuclein caused a marked decrease of cell viability in primary and immortalized neuronal cells. The neuronal cell death appeared to be correlated with the Rab5A-specific endocytosis of α-synuclein that subsequently caused the formation of Lewy body-like intracytoplasmic inclusions. This was further supported by the fact that the expression of GTPase-deficient Rab5A resulted in a significant decrease of its cytotoxicity as a result of incomplete endocytosis of α-synuclein.

Parkinson's disease (PD)[1] is pathologically characterized by progressive degeneration and death of dopaminergic neurons in the substantia nigra. With poorly understood molecular pathogenesis, accumulating evidence points to the central role of α-synuclein aggregates in this disease. α-Synuclein is a major component of Lewy bodies (LBs) and associated Lewy neurites (LNs), pathological hallmarks of PD and dementia with Lewy bodies (1, 2). Two missense mutations in the α-synuclein gene have been identified in some families with early-onset inherited PD (3).

α-Synuclein has also been found in other neuronal and glial inclusions, such as Lewy body-like glial cytoplasmic inclusions (GCIs) of multiple system atrophy (MSA) (4, 5). In addition, α-synuclein is found in the astrocytes and Schwann cells in the motor neuron disease amyotrophic lateral sclerosis (ALS) (4). Many recent findings also suggest that the extracellular aggregation of non-Aβ component of senile plaque, the fibrillogenic fragment of α-synuclein, in the brain may be involved in the pathogenesis of Alzheimer's disease (AD) (6–8). However, the functional roles of α-synuclein in the pathogenesis of these neurodegenerative diseases are unknown.

Endocytosis enables cells to modify and degrade molecules within a continuum of morphologically and biochemically distinct vacuolar compartments. These compartments include early endosome, late endosomes, and lysosomes, which have different capabilities for processing substrates by proteolysis (9). The neuronal endocytic pathway is a finely controlled and efficient intracellular trafficking system for internalizing and processing extracellular nutrients and trophic factors, for recycling or catabolizing receptors and other integral membrane proteins after neurotransmitter release, and for directing information to intracellular biosynthetic pathways.

The early endosome is the first vacuolar compartment along the endocytic pathway. Enhanced endocytic activity, coupled with increased trafficking to endosomes of proteases, was proposed to be a potential mechanism by which β-amyloidogenesis may become accelerated in sporadic AD (10). Rab5, a small GTPase, has been localized to the early endosome and is involved in the regulation of the endocytosis in association with Rabaptin-5 (11–13). Rab5 and Rabaptin-5 have been shown to play a crucial role in neuronal endocytosis (14, 15). In the human brain, Rab5 have been recently identified in neurons (10, 16).

Recent findings that α-synuclein is secreted into cerebrospinal fluid (CSF), and freshly prepared or aggregates of α-synuclein induce cell death in human neuroblastoma SH-SY5Y cells (17, 18) suggest that α-synuclein secreted into or present in extracellular space may exert its cytotoxic effect to neighboring neuronal cells (17). Likewise, when the excessive amounts of α-synuclein accumulates inside neurons, which eventually die, it could be possible that its aggregates leak out of the dead neurons and spread its cytotoxic effect to the neighboring cells (17). On this basis here we investigated the neurotoxicity triggered by exogenous α-synuclein and its pathogenic mechanism. The present study indicated that Rab5-dependent endocytosis of α-synuclein causes LB-like intracytoplasmic inclusions subsequently leading to cell death in neuronal hippocampal cells. These findings may give deeper insights into the pathogenic mechanism of some neurodegenerative diseases caused by the abnormal accumulation of α-synuclein aggregates.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM) and Geneticin were purchased from Life Technologies Inc.

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[1] The abbreviations used are: PD, Parkinson's disease; Aβ, amyloid-β; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APP, Aβ precursor protein; CM-H2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; CSF, cerebrospinal fluid; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; GCI, glial cytoplasmic inclusion; JNK, c-Jun N-terminal kinase; LB, Lewy body; MSA, multiple system atrophy; MTT, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, non Aβ component; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DCF, 2′,7′-dichlorofluorescein.

(Grand Island, NY). Plasmid to express wild type α-synuclein was provided by R. Jakes (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK). α-Synuclein was overexpressed in *Escherichia coli*, and recombinant proteins were purified as previously described (19, 20). Plasmids to express wild type (pEF-BOS-Rab5A) and GTPase-deficient mutant Rab5A (pEF-BOS-Rab5A/S34N and pEF-BOS-Rab5A/Q79L) were provided by K. Iwata (University of Tokyo, Tokyo, Japan).

Cell Culture and Transfection—Rat neuronal hippocampal progenitor H19-7 cells were generated by transduction with retroviral vectors containing the temperature-sensitive SV40 large T antigen, which is functionally active at 33 °C and inactive at 39 °C (21). The H19-7 cells were grown in DMEM containing 10% fetal bovine serum and maintained at 33 °C under 200 μg/ml G418 selection. Prior to treatment with reagent, cells were shifted to 39 °C in N2 medium for 2 days. Transient transfections were performed using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions. A plasmid pCMV-EGFP (CLONTECH), which contains the jellyfish green fluorescence protein gene driven by the cytomegalovirus promoter, was used as an internal control to assess transfection efficiency.

Assessment of Cell Survival by MTT Extraction Assay—Quantitation of cell survival was done using the tetrazolium salt-extraction method (22). 62.5 μl of the 5 mg/ml stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well containing 250 μl of medium in a 24-well plate. After 2 h of incubation at 37 °C, 250 μl of extraction buffer containing 20% sodium dodecyl sulfate and 50% *N*,*N*-dimethyl formamide, pH 7.4, was added. After incubating reagents overnight at 37 °C, the optical density was measured at 570 nm using a Spectra MAX 340 enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA), with extraction buffer used as a blank.

Western Blot Analysis with Anti-ERK, -JNK, or -p38 Antibody—Western blot analysis was performed using anti-ERK and -phosphoERK, anti-JNK and -phosphoJNK, or anti-p38 and -phosphop38 antibody, as described previously (23). The endogenous and activated mitogen-activated protein kinase bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

Immunohistochemistry—Cells were fixed for 10 min in 3.7% formaldehyde in phosphate-buffered saline (PBS). After washing with PBS twice, the cells were incubated for 10 min in 2% Triton X-100 in PBS and blocked with an approximate dilution of primary antibody at 37 °C for 1 h. After washing with PBS twice, secondary antibody conjugated to FITC or rhodamine was added to the samples at 37 °C, and incubated for 1 h. The fixed cells were washed several times with PBS, and mounted in Mowiol (Calbiochem). Immunostained cells were observed using confocal microscopy.

Measurement of ROS Generation—Intracellular ROS production was measured as described elsewhere (24). After stimulation, cells were washed with HEPES-buffered saline and incubated in the dark for 5 min in HBS containing 5 mM of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Molecular Probes). CM-H2DCFDA is hydrolyzed to DCFH in the cell, and DCFH is oxidized to form highly fluorescent DCF in the presence of the correct oxidant. ROS generation was detected as a result of DCFH oxidation. Cells were then examined using confocal microscopy (Leica, TCS NT system). The effect of DCFH photo-oxidation was minimized by collecting the fluorescent image with a single scan (total scan time was 5 s).

Cell Fractionation—After stimulation, cells were washed in ice-cold PBS, lysed via the addition of hypotonic buffer (40 mM HEPES, 4 mM EDTA, 2 mM EGTA, 10 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and incubated for 30 min at 4 °C. Cells were sonicated twice for 10 s with a W-385 sonic oscillator (50% duty cycle, 2-s pulse cycle time, 3-output level) (Heat Systems Ultrasonic Inc). Membrane fractions were prepared by centrifugation (100,000 × *g* for 1 h at 4 °C) in an Ultracentrifuge (Beckman). The supernatant was collected as a cytosolic fraction, and the pellet was washed with PBS. The final pellet was resuspended in 1% Triton X-100 lysis buffer and incubated for 10 min at 4 °C, and membrane fractions were collected as supernatant following centrifugation (130,000 rpm for 10 min at 4 °C). The protein concentration of each fraction was quantitated using a protein assay kit (Bio-Rad).

Metabolic Labeling with [^{35}S]Methionine—Cells were incubated in prewarmed methionine-free DMEM for 3 h, and labeled with [^{35}S]Met (5 μCi/ml, ICN) for 1 h before stimulation. Cells were subsequently washed with ice-cold PBS and lysed in TLB. Primary antibody was added to cell lysates, and the mixture was incubated for 2 h at 4 °C. 10% of Protein A-Sepharose beads were added to the reaction mixtures and incubated for 1 h. Immunoprecipitated samples were washed three times in lysis buffer and solubilized by boiling samples for 5 min. After electrophoresis, the gel was stained with Coomassie Blue dye, soaked in EN^{3}HANCE solution for 60 min (PerkinElmer Life Sciences), and analyzed by autoradiography.

Measurement of Intracellular Ca^{2+} Mobilization—Ca^{2+} ions were measured using fura-2 loading and fluorescence analysis as described previously (25). The cells were washed twice with a HEPES-buffered solution A (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) and loaded with fura-2 by a 30-min incubation at 37 °C in solution A containing 5 μM AM (Molecular Probes, Eugene, OR). After loading, the coverslip was washed once with solution A and assembled to form the bottom of the perfusion chamber. The chamber was perfused continuously with either Ca^{2+}-containing or Ca^{2+}-free solution A. The Ca^{2+}-free solution was prepared by replacing $CaCl_2$ with 3 mM EGTA. The osmolarity of all solution was adjusted to 310 mosM with the major salt prior to use. Fluorescence was measured and calibrated using a PTI system (PTI Delta Ram, New Brunswick, NJ). fura-2 fluorescence was excited at 355 and 380 nm and calibrated by exposing the cells to solutions containing high and low concentration of Ca^{2+} and 10 μM ionomycin.

Electron Microscopy—The samples were fixed with 0.1 M cacodylate buffer (pH 7.4), including 2% glutaraldehyde, 2% paraformaldehyde, and 0.5% $CaCl_2$ for 6 h and washed with 0.1 M cacodylate buffer. The samples were post-fixed with 1.33% OsO_4 in cacodylate buffer for 2 h. Fixed samples were washed with 0.1 M cacodylate buffer for 10 min. After washing, samples were dehydrated with alcohol and incubated with propylene oxide for 10 min. Embedding Epon mixture (Epon812, MNA, DDSA, and DMP30) was prepared, and samples were sectioned using Ultramicrotome followed by double staining with uranyl acetate and lead citrate. Samples were observed by transmission electron microscopy (Philips CM-10).

RESULTS

Induction of Cell Death by α-Synuclein—We examined the effect of various doses of α-synuclein on cell viability in immortalized hippocampal H19-7 cells. In human brain homogenates α-synuclein comprises ~0.5–1% of the total proteins (7). When α-synuclein measuring 5–30 μM was added for 24 h to differentiated H19-7 cells induced by basic fibroblast growth factor, the cell viability decreased in a dose-dependent manner, as measured by MTT extraction assay (Fig. 1*A*). When we counted trypan blue-excluding viable cells or measured the levels of lactate dehydrogenase released from membrane-damaged cells, a similar level of cell death was observed (data not shown). Similar to H19-7 cells, the exposure of primary cortical neurons to α-synuclein for 24 h reduced the cell viability in a dose-dependent manner (Fig. 1*A*). Interestingly, when bacterial Ala-30 → Pro or Ala-53 → Thr α-synuclein, whose mutation was reported in the pathogenesis of familial PD (3), was added to the cells, a similar level of cell death was observed, compared with that by wild type α-synuclein (data not shown). As a control, addition of bacterial glutathione *S*-transferase prepared in the same recombinant expression system had no effect on the cell viability in H19-7 cells (data not shown), suggesting that the induction of cell death is a specific effect of α-synuclein.

Intracellular Response of H19-7 Cells to α-Synuclein—To clarify the intracellular downstream signaling cascades of α-synuclein, we measured the activity of endogenous mitogen-activated protein kinases, such as extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. When 10 μM α-synuclein was added, a significant increase of JNK (Fig. 1*B*) and a lower but marked activation of ERK and p38 activity (data not shown) were observed using anti-phospho-ERK, anti-phospho-JNK, and anti-active p38 antibodies. Some researchers have suggested the involvement of oxidative stress in the pathogenesis of nigral neuronal cell death in PD (26). By using reactive oxygen species-specific fluorescent dye, CM-H2DCFDA, we found that the exogenous addition of α-synuclein causes the formation of a large amount

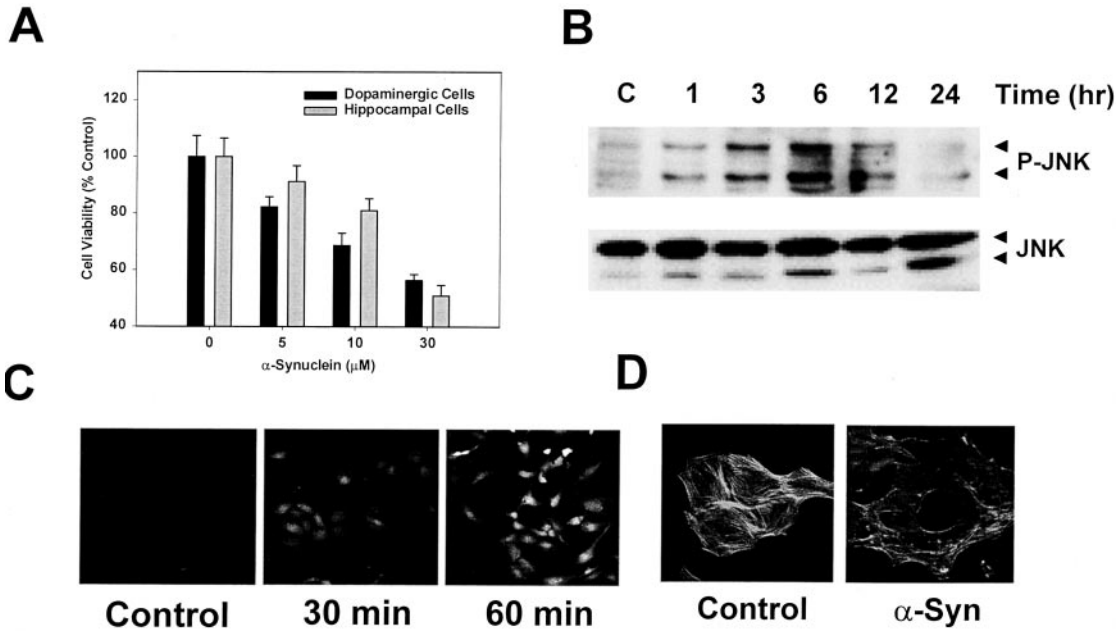

FIG. 1. **Induction of neuronal cell death by α-synuclein.** *A*, cytotoxicity of α-synuclein in neuronal cells. Differentiated H19-7 cells and cortical neuronal cells were stimulated with the indicated concentration of α-synuclein for 24 h. Cell viability, measured using the MTT extraction assay, was defined as 100% of control. Values are represented as means ± S.D. ($n = 3$). *B*, activation of JNK by exogenous α-synuclein in H19-7 cells. The differentiated H19-7 cells were treated with 10 μM α-synuclein for the time indicated. Cell extracts were resolved on 12.5% SDS-polyacrylamide gel electrophoresis and analyzed by Western analysis using antibodies specific to phosphorylated JNK (*P-JNK*). To ensure that equal amounts of protein were loaded, the amounts of non-activated JNK were measured. *C*, ROS generation by the extracellular addition of α-synuclein in H19-7 cells. After stimulation with 10 μM α-synuclein for the time indicated, H19-7 cells were washed with HEPES-buffered saline and incubated in the dark for 5 min in HBS containing 5 mM CM-H2DCFDA. The ROS generation was detected using confocal microscopy. *D*, disruption of intracellular actin structures by exogenous α-synuclein in H19-7 cells. After stimulation with 10 μM α-synuclein for 6 h, cells were fixed in 3.7% formaldehyde. The cells were subsequently incubated with actin antibody followed by secondary antibody conjugated to FITC. The fixed cells were mounted in Mowiol and were observed using confocal microscopy.

of reactive oxygen species for 1 h post-α-synuclein stimulation (Fig. 1*C*), and increasing concentrations of α-synuclein increases the fluorescence (data not shown). In addition, the disruption of cellular cytoskeletal actin structures was observed after 6 h of α-synuclein stimulation (Fig. 1*D*).

Intracellular Transport of Exogenous α-Synuclein—To elucidate how exogenous α-synuclein exerts an intracellular cytotoxic effect, cell fractionation was performed to localize proteins inside the cells. After soluble and particulate fractions had been prepared, each fraction was assayed for the presence of α-synuclein. The control cells were not found to contain any significant levels of α-synuclein (Fig. 2*A*), which is consistent with a previous report in which α-synuclein only begins to be expressed after the postnatal period in the rat central nervous system (27). Most of the α-synuclein was localized in the intracellular soluble fraction but not in the particulate membrane fraction. Interestingly, α-synuclein began to accumulate in the soluble fraction as soon as 1 min after the onset of treatment (Fig. 2*A*). By using metabolic labeling with [^{35}S]Met, it was possible to determine that new protein synthesis of α-synuclein is not observed in the cells after exogenous addition of α-synuclein (Fig. 2*B*). This indicated that exogenous α-synuclein is rapidly transported into H19-7 cells.

Formation of Cytoplasmic Lewy Body-like Inclusions by α-Synuclein—Immunocytochemical visualization of the cells clearly showed that the distribution of α-synuclein was uneven and took the form of granular aggregates in the cytoplasm (Fig. 2*C*). Immunostaining for ubiquitin, synaptophysin, and tau protein are frequently used to visualize Lewy pathology in human brain (29–33), whereas a situation in which microtubule-associated protein tau and α-synuclein were found in the same neuron but not the same parts of the cell has been reported (28). α-Synuclein also binds to tau and stimulates the protein kinase A-catalyzed tau phosphorylation (34). As shown by immunoprecipitation assay, transported α-synuclein binds to various LB proteins, such as synaptophysin, ubiquitin, and tau protein (Fig. 3*A*). Double immunolabeling studies confirmed that virtually all of the α-synuclein-positive aggregates in H19-7 cells were also ubiquitin-positive (Fig. 3*B*). Furthermore, α-synuclein-positive granular deposits included synaptophysin (Fig. 3*C*), as well as tau proteins (Fig. 3*D*), inside the H19-7 cells. Taken together, these results indicate that the components of α-synuclein-positive intracytoplasmic inclusions in H19-7 cells are very similar to those of LB inclusions in PD.

No Change in Membrane Integrity after Addition of Exogenous α-Synuclein—By using electron microscopy, the cell morphologies of control cells and the cells stimulated with α-synuclein were compared. As shown in Fig. 4, the membrane integrity seems to be well-maintained 30 min after the addition of α-synuclein. Furthermore, the finding, that any phenotypic changes characteristic of apoptotic cell death such as nuclear condensation and DNA fragmentation were not observed, implies that necrotic cell death may occur inside the cells caused by extracellular α-synuclein. The most characteristic feature of the cells to which exogenous α-synuclein was added, is the formation of microvilli in the outer plasma membrane. A possible time-dependent endocytosis, via the formation of granules, in which α-synuclein could be enclosed, was observed around the plasma membrane. These data support the previous findings in which α-synuclein is readily transported into the cells by endocytosis.

The highly toxic C-terminal 105-amino acid fragment of amyloid-β (Aβ) precursor protein (APP) can exhibit direct neurotoxic effects by forming Ca^{2+}-permeable cation channels on neuronal membranes (35). To test the possibility that α-synuclein may also change the integrity of cell membrane, resulting in increased intracellular ion transport, intracellular calcium mobilization was measured in the absence or presence

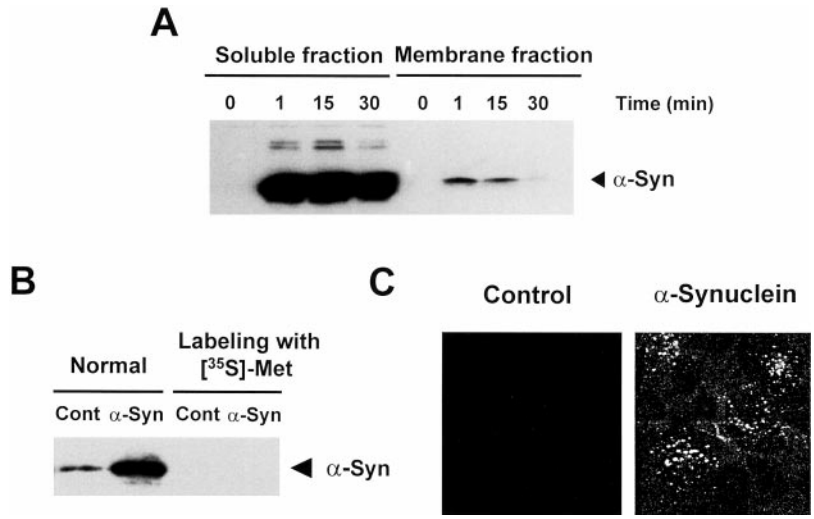

FIG. 2. **Intracellular endocytosis of exogenous α-synuclein.** *A*, intracellular localization of exogenous α-synuclein. After stimulation with 10 μM α-synuclein for the time indicated, membrane and soluble fractions were prepared by cell fractionation. The levels of α-synuclein in each fraction were measured by Western analysis. *B*, intracellular transport of α-synuclein in H19-7 cells. The cells were incubated in methionine-free medium for 3 h and labeled with 5 μCi/ml [^{35}S]Met for 1 h. After stimulation with 10 μM α-synuclein for the time indicated, an antibody to α-synuclein was added to cell lysates and incubated for 2 h at 4 °C. 10% of Protein A-Sepharose beads are added to reaction mixtures and incubated for 1 h. Immunoprecipitated samples were analyzed using autoradiography. *C*, intracellular granular accumulation of exogenous α-synuclein in H19-7 cells. After stimulation with 10 μM of exogenous α-synuclein for 24 h, the cells were immunostained with an antibody specific to α-synuclein.

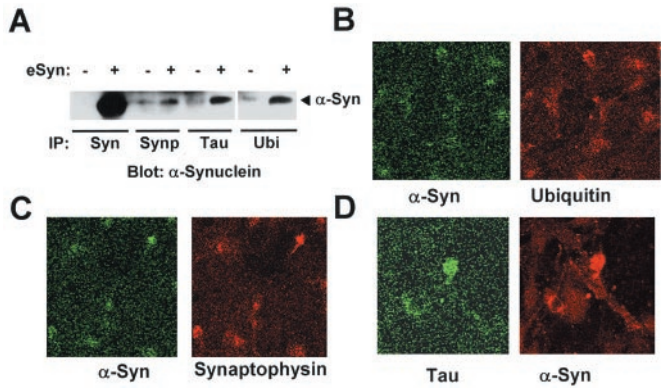

FIG. 3. **Specific binding of α-synuclein to LB proteins, such as ubiquitin, synaptophysin, or tau protein, and intracellular co-localization with α-synuclein.** *A*, cells were either untreated or stimulated with 10 μM α-synuclein (*eSyn*) for 3 h, and the cell lysates were immunoprecipitated against antibodies specific to α-synuclein, synaptophysin (*Synp*), tau (*Tau*), or ubiquitin (*Ubi*), subsequently followed by Western blot analysis using anti-α-synuclein antibody, as indicated. *B–D*, after stimulation with 10 μM exogenous α-synuclein for 3 h, cells were immunostained with an antibody specific to α-synuclein, ubiquitin (*B*), synaptophysin (*C*), and/or tau protein (*D*), respectively, as indicated. After washing the cells, secondary antibody conjugated to FITC or rhodamine was added to the samples at 37 °C for 1 h. The fixed and stained cells were observed by using confocal microscopy.

of α-synuclein. Consistent with previous electron microscopic observations, any change in basal-induced (Fig. 5*A*) as well as *N*-methyl-D-aspartate- or purinergic agonist ATP-induced calcium influxes (data not shown) was not observed up to 15 min in the presence of α-synuclein. When the calcium transport assay was extended to longer time points (30 and 60 min), there was no significant change of intracellular calcium levels (data not shown). These data suggest that the membrane integrity of H19-7 cells appears to be maintained at least in the early stages of cell death induced by α-synuclein.

Identification of a Possible "Receptor" Protein for the Endocytosis of α-Synuclein—To assess whether any membrane protein(s) is involved in the endocytosis of α-synuclein, the cells were treated with varying concentrations of trypsin during cell splitting. Treatment of trypsin at the concentration range of 5–100 μg/ml caused a dose-dependent reduction in cell toxicity by later stimulation with α-synuclein and its endocytosis. Treatment of cells with 250 μg/ml trypsin completely blocked the endocytosis of exogenous α-synuclein (Fig. 5*B*). Given that trypsin does not penetrate the cell membrane and digests only protein domains on the outer surface, this finding supports the hypothesis that "carrier" or "receptor" protein(s) for α-synuclein is present in the plasma membrane. Such a protein-mediated transport mechanism would be required for the intracellular endocytosis of exogenous α-synuclein and subsequent induction of neuronal cell death. To confirm the possibility of protein-mediated transport of α-synuclein, and to identify the receptor protein(s) in the membrane, bi-functional cross-linking reagent was added after stimulation with the intention of linking α-synuclein to any binding protein(s) in its vicinity. Cross-linking of particulate fractions with dimethylsuberimidate after the stimulation revealed the presence of a specific binding protein to α-synuclein with a molecular size of about 60 kDa (Fig. 5*C*).

Rab5A-dependent Endocytosis of α-Synuclein—In cultured neuronal cells, Rab5, which modulates transport kinetics between the plasma membrane and early endosome, is selectively localized to early endosomes (11, 13, 14). The expression of endocytosis regulatory protein Rab5 and Rabaptin-5 in glial cytoplasmic inclusions (GCIs), was observed in brains with multiple system atrophy (MSA) (36). Oligodendrocytes in MSA appear to be functionally altered with respect to the occurrence of GCIs and aberrant expression of α-synuclein. Based on this finding, Rab5 is speculated to be involved in the endocytosis of α-synuclein. To elucidate the involvement of neuronal early endosomes, immunocytochemical investigations were undertaken with an antibody to Rab5A. It was found that a monomeric GTP-binding Rab5A protein binds to α-synuclein in a specific way (Fig. 6*A*). Immunohistochemical analysis clearly showed that Rab5A is co-localized with α-synuclein in a granular form in the cytosolic area (Fig. 6*B*). Overexpression of GTPase-deficient Rab5A proteins resulted in a significant decrease in the rates of the endocytosis of α-synuclein and subsequent cell death (Fig. 7*A*). After transient transfection of the dominant-negative Rab5A mutant into the cells, the particulate and soluble fractions of cell lysates were prepared. Western blot analysis showed that exogenous α-synuclein was not completely transported into the cytosol, re-

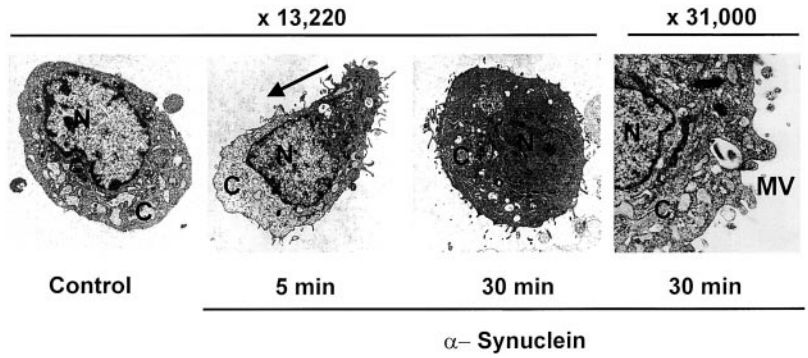

FIG. 4. **Electron microscopic observation of H19-7 cells treated with α-synuclein.** After stimulation with 10 μM α-synuclein for the time indicated, cells were observed by transmission electron microscopy, as described under "Experimental Procedures." The *right panel* on the *bottom* is a micrograph of the cells stimulated with 10 μM α-synuclein for 30 min, showing a higher magnification of enlarged structures, including plasma membrane and surrounding endocytic vesicles (*EV*). *C*, cytosol; *N*, nucleus.

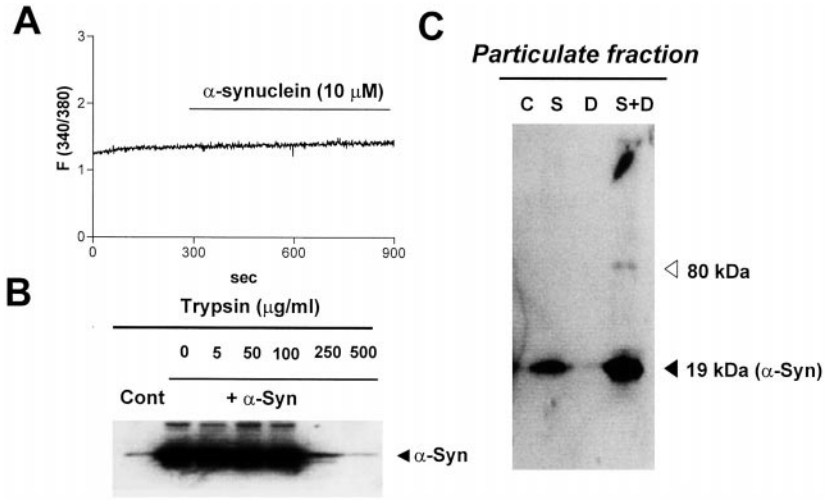

FIG. 5. **Identification of a plausible "receptor" protein for the endocytosis of α-synuclein.** *A*, effect of α-synuclein on intracellular calcium transport in H19-7 cells. The cells were preloaded with fura-2 and perfused with calcium containing HEPES-buffered solution to establish basal Ca^{2+} levels. The cells were subsequently treated with 10 μM α-synuclein, up to 15 min. Each *trace* is from an individual cell in a field of cells and represents the typical response of three independent experiments. *B* and *C*, presence of possible carrier protein for intracellular endocytosis of α-synuclein in H19-7 cells. After reaching ~80% cell confluence, H19-7 cells were detached from the culture dish and treated with the concentration of trypsin indicated for 1 h before re-plating. After incubating the cells with 10 μM α-synuclein for 1 h, the membranous particulate fraction was prepared. The levels of α-synuclein were measured by Western blot analysis (*B*). Cells were either unstimulated (*Co*) or treated with 100 μM dimethylsuberimidate (*D* or *DM*) for 1 h in the absence or presence of 10 μM α-synuclein (*S*). The particulate cell lysates were isolated, and the levels of, and any protein(s) interacting with, α-synuclein were examined by Western analysis (*C*).

sulting in the decreased accumulation of α-synuclein in the cytosol, compared with that of control cells (Fig. 7*B*). On the contrary, higher levels of exogenous α-synucleins were anchored in the membrane fraction, suggesting that Rab5A is required for the intracellular transport of exogenous α-synuclein (Fig. 7*C*). These data implied that Rab5A is critical for the complete endocytosis of exogenous α-synuclein followed by induction of neuronal cell death.

DISCUSSION

Although α-synuclein is highly abundant in presynaptic terminals and in cytoplasmic Lewy bodies, human α-synuclein was originally isolated from extracellular plaques of AD brains as a 19-kDa protein precursor of the highly hydrophobic 35-amino acid metabolite, non-Aβ component (NAC) of senile plaques (6, 37). The NAC peptide can self-aggregate into fibrils and induces aggregation of Aβ peptide in extracellular space (2, 38). The NAC fragment comprises about 10% of the protein that remains insoluble after SDS detergent extraction of β-amyloid plaques (6). Therefore, though the precise mechanism of how α-synuclein is digested into NAC and subsequently aggregated into plaques is not clarified, it would be possible that α-synuclein accumulates in an extracellular space under some pathogenic conditions, such as PD or AD. Likewise, if the excessive amounts of α-synuclein accumulate inside neurons, which eventually die, it could be possible that its cytoplasmic aggregates leak out of the dead neurons and spread its cytotoxic effect to the neighboring cells (17). A recent finding (18) that full-length α-synuclein is present in CSF from PD and normal subjects implicated that α-synuclein is released by neurons in the extracellular space as part of its normal cellular processing or, alternatively, as an effect of neuronal degeneration and death. Based on these previous finding, we have investigated the pathogenic mechanism of exogenous α-synuclein. The present study reveals a possible pathogenic mechanism of intracellular LB-forming neurodegenerative diseases triggered by extracellular accumulation of α-synuclein. Excessive levels of α-synuclein accumulated extracellularly transports rapidly into the cells, resulting in neuronal cell death. When we added the protein aggregates of α-synuclein, prepared by the addition of zinc ion (20), its cytotoxic effect at the same concentration was more potent, and the transport of the α-synuclein proteins was markedly increased, compared with that of the freshly prepared α-synuclein.[2]

It should be noted that cells under stress or pathogenic condition show altered translocation of proteins, *i.e.* polyglutamine proteins that are normally cytoplasmic accumulate in the nucleus as aggregates in trinucleotide repeat disease (39). In addition, tau levels in CSF are elevated in AD and other neurodegenerative diseases (40, 41). In a similar way, brain and spinal cord degenerating neurons are also implicated as a

[2] J. Y. Sung and K. C. Chung, unpublished observation.

source of heavy metal-containing spheroid-like organelles in the CSF of ALS patients (42). Considering their frequently superficial localization in the parenchyma, their release into the CSF with their metal load appears very likely as a step in a possible transport mechanism from the cytoplasm to the extracellular space (43). This raises a more fundamental question: Is the localization change of protein aggregates pathogenic or indicative of a stressed cell? Notwithstanding evidence that aggregates are not likely the first step in pathogenesis, it is striking that neurodegenerative diseases such as AD, ALS, PD, and polyglutamine repeat disease involve the gradual accumulation of mutant proteins in the nuclei, cytoplasm, or extracellular space. Misfolded or otherwise altered in conformation because of mutation, these proteins may all interact with other proteins in an aberrant fashion, undergo altered processing and, possibly, decreased ubiquitin-dependent degradation. The coexistence of α-synuclein with ubiquitin and proteasome subunits in Lewy bodies in sporadic PD disease also suggests that alterations of α-synuclein catabolism may contribute to the pathogenesis of PD.

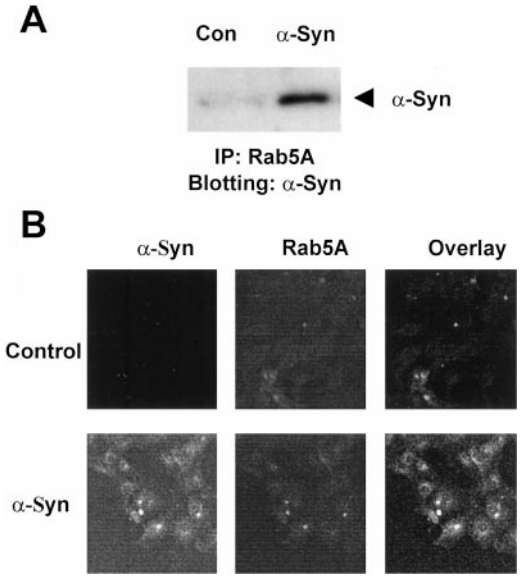

FIG. 6. **Specific binding of α-synuclein to Rab5A and Rab5A-dependent endocytosis of α-synuclein in H19-7 Cells.** *A*, after stimulation with 10 μM α-synuclein for 1 h, H19-7 cells were immunoprecipitated against an antibody specific to Rab5A. Cell lysates were resolved using 12.5% SDS-polyacrylamide gel electrophoresis and blotted with α-synuclein IgG. *B*, after adding 10 μM α-synuclein, the cells were immunostained with an antibody specific to either α-synuclein or Rab5A, as indicated. The immunostained cells were observed using confocal microscopy.

Interestingly, we observed that the decrease of cell viability by wild type and mutant α-synucleins (A30P and A53T) is not largely different. Consistent with our current finding, the pattern of subcellular localization, cytotoxicity, and degradation between wild type and mutant α-synuclein have been reported not to be largely different (44–46), whereas there are several reports of situations in which mutated α-synuclein sensitizes neuronal cells to neurotoxic stimuli, such as oxidative damage (47) and dopamine toxicity (48). In addition, the exogenous addition of wild type or mutant synucleins induces the same level of cell death in neuroblastoma SH-SY5Y cells (17). Furthermore, two mutations within the α-synuclein originally identified in one Italian-American and three Greek kindreds with familial PD (3) have not been found from any other broad white and Asian population (49–52), suggesting that these missense mutations at the coding region of α-synuclein gene are a very rare cause and not relevant for most patients with sporadic idiopathic PD.

A leading hypothesis about the biochemical pathogenesis of PD centers on the formation of reactive oxygen species leading to oxidative damage to nigral dopaminergic neurons (53). Our study has shown that exogenous synuclein caused significant generation of reactive oxygen species in the cells. It was shown

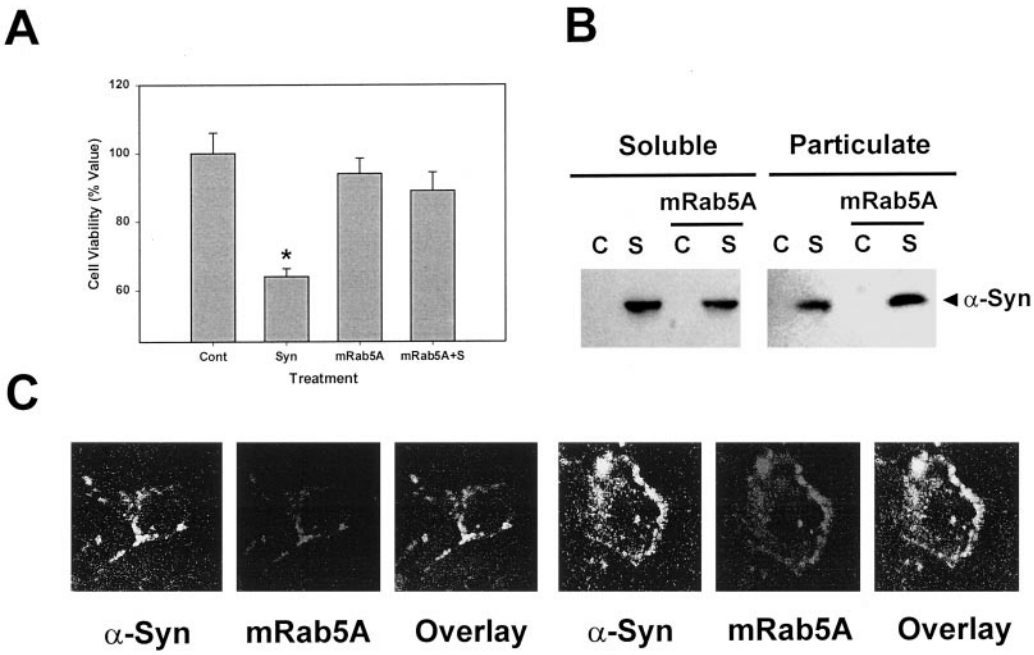

FIG. 7. **Effect of GTPase-deficient Rab5A on the intracellular endocytosis of α-synuclein and its cytotoxicity in H19-7 cells.** *A*, after transient transfection of H19-7 cells with 5 μg of parental vector or plasmid to express GTPase-inactive Rab5A mutant (*mRab5A*), the change in cell viability induced by 10 μM α-synuclein (*Syn* or *S*) was measured using the MTT extraction assay. Data are plotted as the mean as the mean plus the range of sample from three independent experiments. *B*, the levels of α-synuclein in membrane and soluble fractions (*S*) prepared by cell fractionation were examined by Western blot analysis. *C*, after transient transfection with 5 μg of plasmid expressing the GTPase-inactive Rab5A mutant followed by 10 μM α-synuclein for 1 h (*α-Syn*), the cells were immunostained with an antibody specific to either α-synuclein or Rab5A, as indicated. The fixed and stained cells were observed using confocal microscopy.

that nitrated α-synuclein is present in the major filamentous building blocks of PD, dementia with LB, the LB variant of AD, and MSA brain, as well as in the insoluble fractions of affected brain regions of synucleinopathies (53). Post-mortem studies provide ample evidence for increased oxidative stress in the nigra of PD patients, including iron content, impaired mitochondrial function, alterations in the protective antioxidants system as well as suggesting oxidative damage to lipids, proteins, and DNA (54).

There are a few lines of evidence suggesting that α-synuclein may play a role in the membrane transport and synaptic membrane genesis. First, α-synuclein has been reported to bind to rat brain vesicles (55) and synthetic phospholipid membranes (56). Second, α-synuclein was shown to be a highly specific inhibitor of phospholipase D2 (57). This suggests that the level and modification of α-synuclein may affect phospholipase D2 activity, thereby modulating the cleavage of membrane lipids and membrane biogenesis. Third, α-synuclein has been reported to co-localize with synaptic vesicles, suggesting its association with lipids (58).

Although α-synuclein rapidly translocates across the plasma membrane (Fig. 2*A*), the data from rat brain vesicles and synthetic phospholipid membranes suggest that α-synuclein is not sufficient to traverse the membrane, implicating the presence of additional factors that are required for complete endocytosis of α-synuclein. We identified that a plausible carrier protein with 60-kDa molecular size appears to bind to α-synuclein in a specific way and is speculated to play a role in the endocytosis of exogenous α-synuclein. Further characterization of the binding protein for α-synuclein will enhance our understanding of the mechanism of how the cytotoxic signals of exogenous α-synuclein elicit cell death response. In a similar way, a 66-kDa membrane protein was identified that appears to bind prion protein and mediate its cytotoxicity (59).

In the brains with MSA, Rab5 and Rabaptin-5 immunoreactivities were observed in some GCIs (36). Oligodendrocytes in MSA appear to be functionally altered in their nature in terms of the occurrence of GCIs and aberrant expression of neuron specific proteins, such as α-synuclein and microtubule-associated protein 2. Alzheimer APP is localized in nerve terminal preparation to Rab5-containing endocytic trafficking organelles distinct from those implicated in the synaptic vesicle pathway (60). An axonal cell-surface protein, APP, is internalized with recycling synaptic membrane proteins but is subsequently sorted away from synaptic vesicles (61). In addition, the present study revealed that Rab5A plays a critical role during the endocytosis of exogenous α-synuclein followed by the induction of acute cytotoxic effect.

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