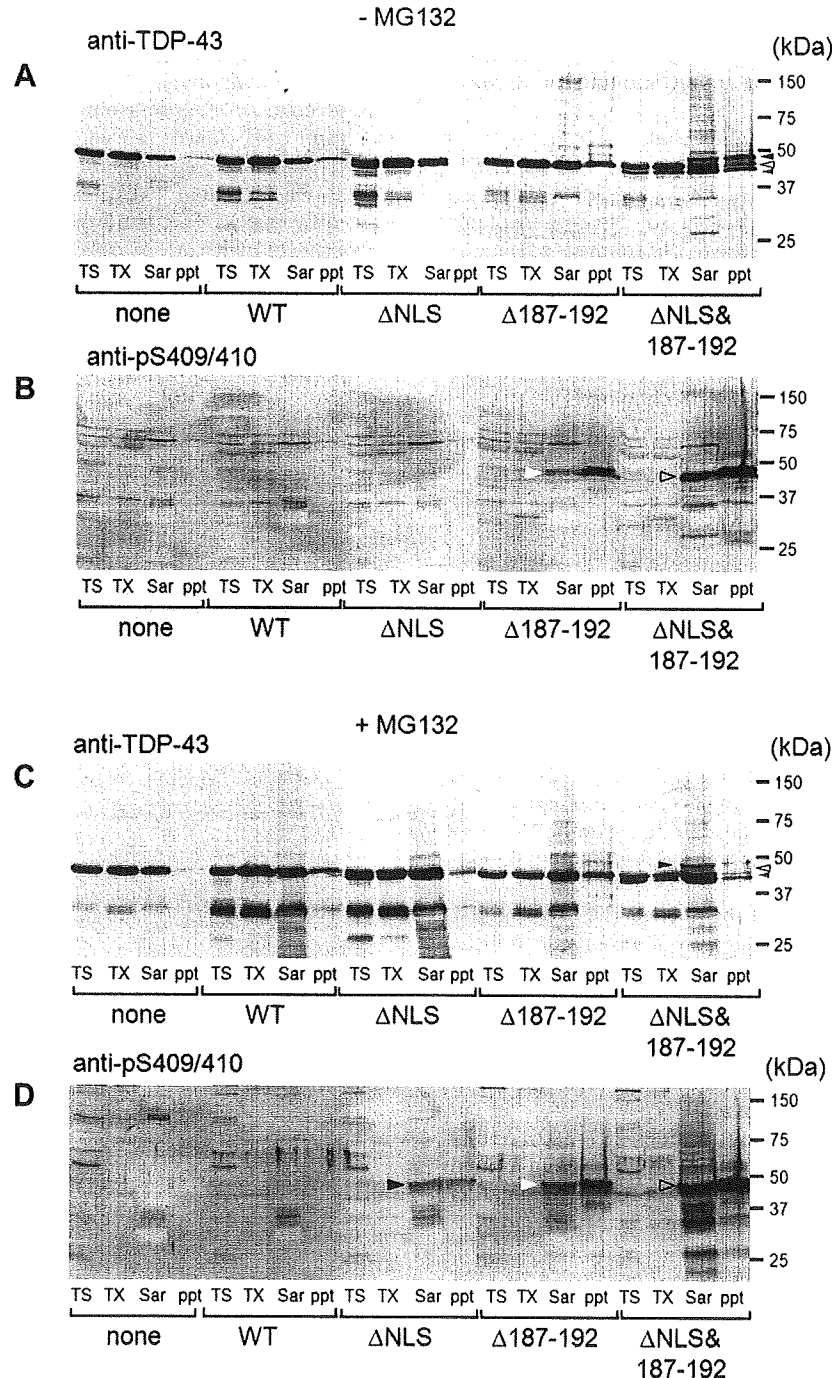


no immunoreactivity for anti-pS409/410 and anti-Ub antibodies in the presence or absence of MG132 (Fig. 3A and B). Cells transfected with  $\Delta 187-192$  showed small dot-like structures positive for anti-pS409/410 antibody but negative for anti-Ub antibody without MG132 treatment (Fig. 3C). Cells transfected with  $\Delta 187-192$  after the MG132 treatment contained inclusion-like structures in nuclei that were intensely labeled with both antibodies (Fig. 3D:  $\sim 8\%$  of inclusion-positive cells). Relatively large inclusions ( $\sim 5 \mu\text{m}$ ) were immunopositive for both antibodies, while small ones ( $< 1 \mu\text{m}$ ) were positive for only phospho-specific TDP-43 antibody, suggest-

ing that abnormal phosphorylation of TDP-43 is an early event in the process of inclusion formation.

Cells transfected with  $\Delta\text{NLS}$  showed no immunoreactivity without MG132 treatment (Fig. 3E), but showed formation of cytoplasmic inclusions positive for both anti-pS409/410 and anti-Ub after MG132 treatment (Fig. 3F:  $\sim 10\%$  of inclusion-positive cells). Furthermore, cells transfected with mutant TDP-43 lacking both the NLS and residues 187-192, named  $\Delta\text{NLS}\&187-192$ , formed intracellular round inclusion-like structures positive for both anti-pS409/410 and anti-Ub antibodies, independently of treatment



**Fig. 4.** Immunoblot analyses of intracellular inclusions of deleted TDP-43 mutants in SH-SY5Y cells. Untransfected cells (none) and cells 72 h post transfection with wild-type (WT) or TDP-43 deletion mutants, before (A and B) and after (C and D) treatment with MG132 ( $1 \mu\text{M}$  overnight), were sequentially extracted with Tris-saline (TS), 1% Triton-X (TX) and 1% Sarkosyl (Sar), and these supernatants and the Sarkosyl-insoluble pellets (ppt) were run on SDS-PAGE, transferred to PVDF membrane and probed with anti-TDP-43 antibody (A and C) and anti-pS409/410 (B and D).

with MG132 (Fig. 3G: ~8% of inclusion-positive cells and H: ~12% of inclusion-positive cells). The inclusions were ~10  $\mu$ m in diameter, being very similar in size to the neuronal cytoplasmic inclusions found in patients with FTL-D-U [2].

### 3.3. Immunoblot analysis of intracellular inclusions in the cultured cell models

Cells expressing wild-type or mutant TDP-43 were sequentially extracted, and the supernatants and pellets were analyzed by immunoblotting. On analysis of cell lysates using anti-TDP-43 antibody, endogenous TDP-43 at 43 kDa was detected in all fractions. Immunoreactivity of it was the strongest in TX-soluble fraction, and was the weakest in Sar-insoluble fraction (black-lined arrowheads in Fig. 4A and C). A similar band pattern but with stronger immunoreactivities was detected in cells transfected with wild-type TDP-43 (WT).

The  $\Delta$ NLS mutant TDP-43, which was detected as bands with slightly lower molecular weight than that of the wild-type, was also recovered mostly in the TS- and TX-soluble fractions (Fig. 4A). The intensities of bands in the Sar-soluble and -insoluble fractions were slightly increased after MG132 treatment (Fig. 4C). Interestingly, these were positive for anti-pS409/410 (a black arrowhead in Fig. 4D), suggesting that inhibition of proteasome activity induces the aggregation of phosphorylated  $\Delta$ NLS.

In contrast, expression of  $\Delta$ 187-192 mutant TDP-43, which was detected as a band with almost the same molecular weight as that of endogenous TDP-43, resulted in significant increases in the Sar-soluble and -insoluble (ppt) fractions as compared with those in cells transfected with wild-type TDP-43 (Fig. 4A). Smear and higher-molecular-weight bands were also detected in the Sar-soluble and -insoluble fractions (Fig. 4A). We also observed pS409/410-positive bands in the Sar-soluble and -insoluble fractions in the absence or presence of MG132 (white arrowheads in Fig. 4B and D).

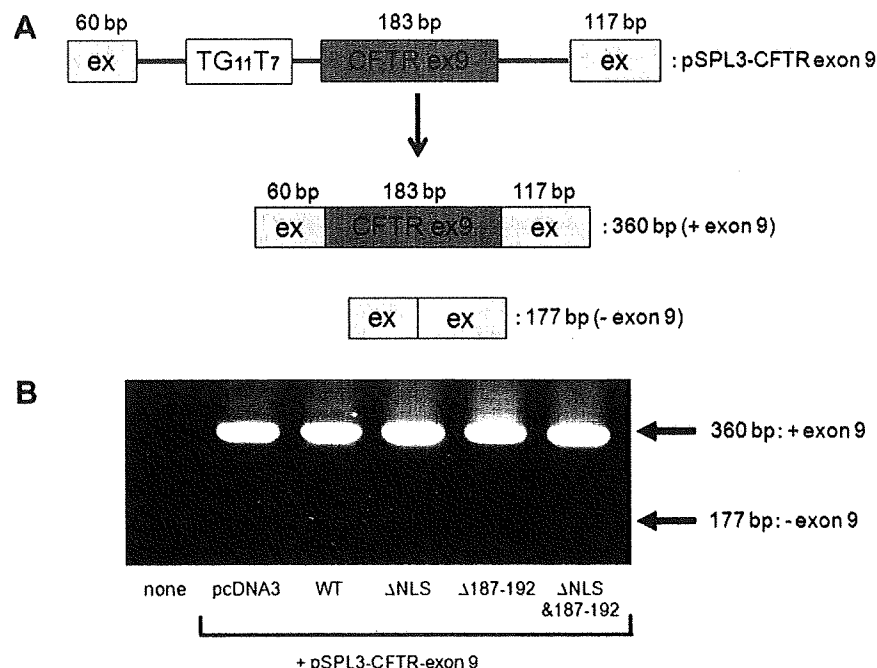
Finally, immunoblots of lysates from cells expressing  $\Delta$ NLS&187-192 mutant TDP-43 (~41 kDa bands marked with grey arrowheads in Fig. 4A and C) showed high-molecular-weight bands of ~45 kDa (black arrowheads in Fig. 4A and C) and smears in the Sar-soluble and -insoluble fractions. The bands of ~45 kDa, smears, and C-terminal fragments at 25-37 kDa were highly immunoreactive with anti-pS409/410 antibody independently of MG132 treatment (black-lined arrowheads in Fig. 4B and D). These were similar characteristic band patterns to those found in immunoblot analyses of brain lysates of FTL-D-U and ALS as previously reported [6].

### 3.4. Deletion mutants of TDP-43 lost the exon skipping activity

To evaluate the functional significance of the deletion mutants of TDP-43 used in this study, we performed CFTR exon 9 skipping assay. As shown in Fig. 5B, mRNA from cells transfected with empty vector pcDNA3 gave only one RT-PCR band of 360 bp, while that from cells transfected with pcDNA3-TDP-43 wild-type gave two RT-PCR bands, 360 and 177 bp, showing that skipping of CFTR exon 9 was increased by expression of wild-type TDP-43. In contrast, only one RT-PCR band of 360 bp was observed from cells co-transfected with  $\Delta$ NLS,  $\Delta$ 187-192, or  $\Delta$ NLS&187-192, indicating that these mutants do not have skipping activity of CFTR exon 9, which is one of known physiological functions of TDP-43.

## 4. Discussion

In this study, using SH-SY5Y cells and a phosphorylated TDP-43 specific antibody established by ourselves, we examined the effect of deletion of two candidate sequences for NLS, residues 78-84 and 187-192 of TDP-43, and proteasomal inhibition on inclusion formation. Mislocalization of TDP-43 into cytoplasm caused by deletion of residues 78-84 proves that this sequence indeed functions as NLS. This result is largely consistent with the previous report by Winton et al., which showed that residues 82-98 were



**Fig. 5.** CFTR exon 9 skipping assay of the deletion mutants of TDP-43. (A) Schematic diagram of the reporter plasmid pSPL3-CFTR exon 9. This plasmid contains the repeat sequence of TG11T7 in which the TG11 repeat is recognized by TDP-43, causing the CFTR exon 9 to be spliced out. The insert of this plasmid contains two exons of HIV-1 tat gene (60 and 117 bp, respectively; light grey boxes) flanking CFTR exon 9 (183 bp; a dark grey box). RT-PCR is expected to generate two products with (360 bp) and without CFTR exon 9 (177 bp). (B) Gel electrophoresis of RT-PCR products of RNA from transfected cos-7 cells. The RNAs from cos-7 cells, co-transfected with the reporter plasmid pSPL3-CFTR exon 9 plus pcDNA3 expression vectors were used as templates for RT-PCR analysis. The products were analyzed by electrophoresis in 1.5% agarose gel.

required for TDP-43 entry into the nucleus [7]. Formation of intranuclear TDP-43 positive dot-like structures caused by deletion of residues 187–192 suggests that this sequence does not function as a NLS but is nonetheless important to maintain a physiological state of TDP-43 in the nucleus. Loss of the exon skipping activity of CFTR exon 9 observed in cells transfected with this mutant may also support such a notion.

The results of the present study suggest that mislocalization of TDP-43 in cytoplasm is not a sufficient condition for aggregation of TDP-43, since the treatment of MG132, a proteasomal inhibitor, is needed to cause the formation of inclusion in cells transfected with a mutant TDP-43 lacking residues 78–84. Proteasome inhibition also induced formation of intranuclear inclusions in cells transfected with a mutant TDP-43 lacking residues 187–192. These results also suggest that a proteasome activity plays an important role for degradation of TDP-43. Impairment of the ubiquitin-proteasome system has recently been suggested to be related to the onset of neurodegenerative diseases. For instance, Bence et al reported that intracellular aggregates of a huntingtin fragment containing a pathogenic polyglutamine repeat directly impaired the function of the ubiquitin-proteasome system [9]. Keck et al., showed that proteasome was inhibited by paired helical filament-tau in brains of patients with Alzheimer's disease [10]. It should be further investigated whether the proteasome activity is actually decreased in brains of patients with TDP-43 proteinopathies, as Keller et al., reported that a significant decrease in proteasome activity was observed in AD brains [11]. Function of autophagy-lysosome degradation system may be an issue to be investigated as well, since inhibition of autophagic degradation by depletion of the endosomal sorting complexes required for transport (ESCRT) subunits causes accumulation of TDP-43 in ubiquitinated inclusions in cultured cells [12].

In contrast to a mutant lacking residues 78–84 or 187–192, double-deletion mutant of these sequences caused inclusion formation without proteasomal inhibition in this study. These results suggest the possibility that the double mutant protein has a higher propensity to aggregate than each single mutant protein. In this context, it should be noted that in insoluble fraction from FTLU brains, the amount of C-terminal fragments of TDP-43 is higher than that of the full-length TDP-43 [6]. These findings suggest that conformation or modifications of TDP-43 is another important factor for inclusion formation.

Importantly, intranuclear or cytoplasmic inclusions observed in this study were immunopositive for both a phosphorylation-dependent anti-TDP-43 antibody and anti-Ub antibody, suggesting that those consist of phosphorylated and ubiquitinated TDP-43. Biochemical analyses also support that abnormal phosphorylation of TDP-43 takes place in cells with inclusions. These results suggest that our cellular models recapitulate the phenotypes of TDP-43 proteinopathies both pathologically and biochemically. These

models are expected to be valuable tools for understanding the pathological process underlying TDP-43 proteinopathies and for identifying candidate drugs to prevent intracellular aggregation of TDP-43.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.12.031.

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## Identification of casein kinase-1 phosphorylation sites on TDP-43

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### ABSTRACT

TAR DNA-binding protein of 43 kDa (TDP-43) is deposited as hyperphosphorylated cytoplasmic and intranuclear inclusions in brains of patients with frontotemporal lobar degeneration with ubiquitinated inclusions and amyotrophic lateral sclerosis. In this study, we identified 29 phosphorylation sites on recombinant TDP-43 that are phosphorylated by casein kinase-1 (CK1). Interestingly, 18 of them were located in the C-terminal glycine-rich region of TDP-43. Our results indicate that CK1-mediated phosphorylation may play a role in the pathogenesis of these diseases.

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### Introduction

TAR DNA-binding protein 43 (TDP-43), encoded by the *TARDBP* gene on chromosome 1, is a highly conserved, ubiquitously expressed nuclear protein. It regulates gene transcription, exon splicing, and exon inclusion [1–4], and is also a multifunctional RNA-binding protein [4]. Recently, TDP-43 was identified as a major disease-associated protein in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and in amyotrophic lateral sclerosis (ALS) [5,6]. We previously reported that deposited TDP-43 is abnormally phosphorylated, and several antibodies raised against synthetic phosphorylated TDP-43 peptides recognized the pathological structures in FTLD-U and ALS [7]. Immunoblot analysis with these antibodies showed that the Sarkosyl-insoluble fraction extracted from brains of patients with FTLD-U and ALS contains hyperphosphorylated TDP-43 with a molecular weight of 45 kDa, together with fragments of 18–28 kDa [7,8]. Similar characteristic band patterns were obtained in the case of cultured cells expressing mutant TDP-43 [9]. In an *in vitro* phosphorylation assay of recombinant TDP-43, the product(s) of casein kinase-1 (CK1) phosphorylation showed similar electrophoretic mobility to that of hyperphosphorylated TDP-43 present in the

brains of patients with FTLD-U and ALS, suggesting that CK1 may be involved in the hyperphosphorylation of TDP-43 [7,8]. To date, however, there is little information about the sites of the putative CK1-mediated hyperphosphorylation on TDP-43. In this study, we identified multiple sites on recombinant TDP-43 that are phosphorylated by CK1; in particular, the C-terminal Gly-rich region of TDP-43 appears to have a favorable conformation for CK1-mediated phosphorylation.

### Materials and methods

**TDP-43 construct and *in vitro* phosphorylation of TDP-43.** Human TDP-43 cDNA was subcloned into pRK172 expression vectors and transformed into *Escherichia coli* BL21 (DE3) [7]. Crude extracts from *E. coli* expressing human TDP-43 were applied to a heparin-Toyopearl column. The column was washed, and TDP-43 was eluted with 0.5 M NaCl solution. The eluate (partially purified TDP-43) was phosphorylated with CK1 (New England Biolabs, Beverly, MA) at 30 °C for 14 h.

**SDS-polyacrylamide gel electrophoresis (PAGE) and *in-gel* digestion.** Phosphorylated TDP-43 was separated by SDS-PAGE [7]. The band of phosphorylated TDP-43 was excised and soaked in 50 mM Tris-HCl, pH 8.0 containing 50% acetonitrile for 30 min. The gel was dried in a Speed-Vac (Savant) and incubated in 50 mM Tris-HCl, pH 8.0 containing 125–250 ng of modified trypsin

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(Roche Diagnostics, Mannheim, Germany), chymotrypsin (Roche Diagnostics, Mannheim, Germany), or thermolysin at 37 °C for 6–20 h. The digests were extracted from the gel twice with 100 µl of 0.1% TFA containing 60% acetonitrile. These two extracts were combined, evaporated in a Speed-Vac, and stored at –80 °C until assayed.

**Liquid chromatography-ion trap mass spectrometry (LC-MS/MS).** The sample was resuspended in 0.1% formic acid containing 2% acetonitrile and applied to a Paradigm MS4 (Microm BioResources Inc., Auburn, CA) HPLC system fitted with HTC-PAL automatic sampler (CHROMSYS LLC, Alexandria, VA). A reverse-phase capillary column (Develosil ODS-HG5, 0.075 mm i.d. × 50 mm or 0.075 mm i.d. × 150 mm, Nomura Chemical Co., Ltd., Seto, Japan) was used at a flow rate of 200 or 300 nl/min with a 4–80% linear gradient of acetonitrile. Eluted peptides were directly detected with an ion trap mass spectrometer, LXQ (Thermo Fisher Scientific Inc.) at a spray voltage of 1.9 kV and collision energy of 35%. The

mass acquisition method consisted of one full MS survey scan followed by MS/MS scan of the most abundant precursor ions from the survey scan. Dynamic exclusion for MS/MS spectra was set to 30 s. Furthermore, if neutral loss of 98, 49, or 32.7 Da was detected (corresponding to loss of phosphoric acid from singly, doubly, and triply charged precursor ions) among the most abundant ions in the MS/MS scan, a MS/MS/MS scan was then carried out. The data were analyzed with BioWorks (Thermo Fisher Scientific Inc.) and Mascot (Matrix Science Inc., Boston, USA) software.

**Calcium phosphate precipitation.** Calcium phosphate precipitation was carried out as described [10]. Some extracted digests were dissolved in 44 µl of pure water, which was obtained from an EASYpure system (Barnstead International, Thermo Fisher Scientific Inc.). Then 2 µl of 0.5 M Na<sub>2</sub>HPO<sub>4</sub> and 2 µl of 2 M NH<sub>4</sub>OH were added and mixed, followed by the addition of 2 µl of 2 M CaCl<sub>2</sub>. The solution was vortexed and centrifuged at 18,000g for 30 min. The supernatant was removed, and the precipitate was

**Table 1**  
Identified phospho-peptides obtained from CK1 phosphorylated TDP-43.

Residue	Amino acid sequence	Charge	Mass (actual)	Mass (calculated)
<i>Trypsin digestion</i>				
83–95	RKMDETDASS(p)AVK	2	1516.87	1516.66
83–95	RKM(ox)DET DASS(p)AVK	2	1533.30	1532.65
84–95	KMDETDASS(p)AVK	1	1360.53	1360.65
84–95	KM(ox)DET DASS(p)AVK	2	1377.22	1376.55
85–95	MDET DASS(p)AVK	2	1233.40	1232.46
85–95	M(ox)DET DASS(p)AVK	2	1249.29	1248.46
85–95	M(ox)DET DASS(p)S(p)AVK	2	1329.16	1328.42
85–95	MDET(p)DASSAVK	2	1232.72	1232.46
85–97	MDET DASS(p)AVKVK	2	1461.46	1459.63
115–136	TT(p)EQDLKEYFSTFGVLMVQVK	3	2688.66	2687.24
177–189	LPNSKQS(p)QDEPLR	2	1669.54	1670.71
182–189	QS(p)QDEPLR	2	1051.31	1051.43
228–251	AFAFVFTADDQIAQS(p)LCGEDLIK	2	2694.69	2694.27
252–263	GIS(p)VHISNAEPK	2	1332.31	1330.63
269–275	QLERS(p)GR	2	925.98	924.42
276–293	FGGNPGFGNQGGFGNS(p)R	2	1807.23	1805.73
409–414	SS(p)GWGM	1	703.22	703.20
409–414	S(p)S(p)GWGM(ox)	2	798.90	799.16
<i>Chymotrypsin digestion</i>				
1–27	MS(p)EY(p)IRVTEDENDPEIPISEDDGTVL	2	3254.58	3254.31
5–27	IRVTEDENDPEIPISEDDGT(p)VL	2	2664.68	2664.17
277–299	GGNPGFGFNQGGFGNS(p)RGGGAGLGNNQGSNMGGGMNF	2	2072.66	2070.87
386–401	GS(p)ASNAGSGSGFNGGF	2	1451.54	1452.53
386–401	GSAS(p)NAGSGS(p)GFNGGF	2	1533.92	1532.50
398–414	NGGFGSSMDS(p)KSSGWGM(ox)	2	1787.11	1786.63
<i>Thermolysin digestion</i>				
86–96	DET DASS(p)AVKV	2	1201.54	1200.49
236–249	DDQIAQS(p)LCGEDLI	2	1599.12	1598.65
242–250	S(p)LCGEDLI	1	1117.63	1117.44
270–276	LEERS(p)GR	2	942.12	943.43
295–305	GGAGLGNNQGS(p)	2	1010.45	1010.38
295–308	GGAGLGNNQGS(p)NMG	2	1329.24	1328.48
301–311	NNQGS(p)NMGGGM(ox)	1	1160.60	1160.36
336–345	MMGMLAS(p)QQ	2	1189.72	1189.43
340–347	LASQNNQS(p)	2	952.61	954.38
347–354	SGPS(p)GNN	1	839.62	839.28
364–369	NQAFGS(p)	1	703.56	702.24
369–380	SGNNSYS(p)GSNS	2	1209.19	1209.39
369–380	SGNNSYSGS(p)NS(p)	2	1289.47	1289.36
385–394	WGS(p)AS(p)NAGS(p)	2	1075.07	1075.25
385–396	WGSAS(p)NAGS(p)GSG	2	1196.59	1196.35
387–397	SASNAGS(p)GSGF	2	1020.59	1020.35
391–405	AGS(p)GS(p)GFNGGFGS(p)S(p)M	2	1638.71	1638.39
393–401	SGS(p)GFNGGF	2	906.77	908.31
393–401	S(p)GSGFNGGF	2	907.44	908.31
395–405	SGFNGGFGSS(p)M	2	1126.61	1126.38
397–405	FNGGFGS(p)	2	765.47	765.25
400–410	GFGSS(p)MDSKSS	2	1185.55	1184.41
405–414	MDS(p)KS(p)S(p)GWGM(ox)	2	1340.04	1340.33

p, phosphorylation; ox, oxidation.

washed with 80 mM CaCl<sub>2</sub>. After centrifugation as described above, the washing solution was removed. The pellet was dissolved in 20 μl of 5% formic acid and applied to the LXQ LC-MS/MS as described above.

## Results

Recombinant TDP-43 was phosphorylated with CK1 and the products were purified by means of SDS-PAGE as described [7]. Phosphorylated TDP-43 was digested in the gel with trypsin, chymotrypsin and thermolysin. On LC-MS/MS analysis with LXQ, 18, 6, and 23 phosphorylated peptides were obtained from the trypsin, chymotrypsin, and thermolysin digests, respectively (Table 1). MS/MS analysis data for two representative peptides are shown in Fig. 1. A sequence coverage of 98% was achieved for TDP-43; residues 80–82 and 264–270 were not covered (data not shown).

We identified 29 phosphorylation sites, including the previously reported Ser 91 and Ser 92 [11], as shown in Fig. 2. Interestingly, 18 of these sites were located in the C-terminal glycine-rich region (274–414) of TDP-43 (Fig. 2).

The NetPhosK 1.0 server produces neural network-based predictions of kinase-specific eukaryotic protein phosphorylation sites [12,13]. Prediction scores of CK1 phosphorylation for all serine and threonine residues in TDP-43 were calculated with this server. The scores and the actual phosphorylated sites identified as described above are summarized in Table 2. Although no significant difference of the distribution of the prediction score was seen between the 1–273 region and the 274–414 region (C-terminal Gly-rich region), CK1 clearly phosphorylated more serine/threonine residues in the 274–414 region. In particular, 12 of 14 residues in the low score range (0.36–0.39) were phosphorylated in the 274–414 region, comparing with 6 of 27 residues in the 1–273 region.

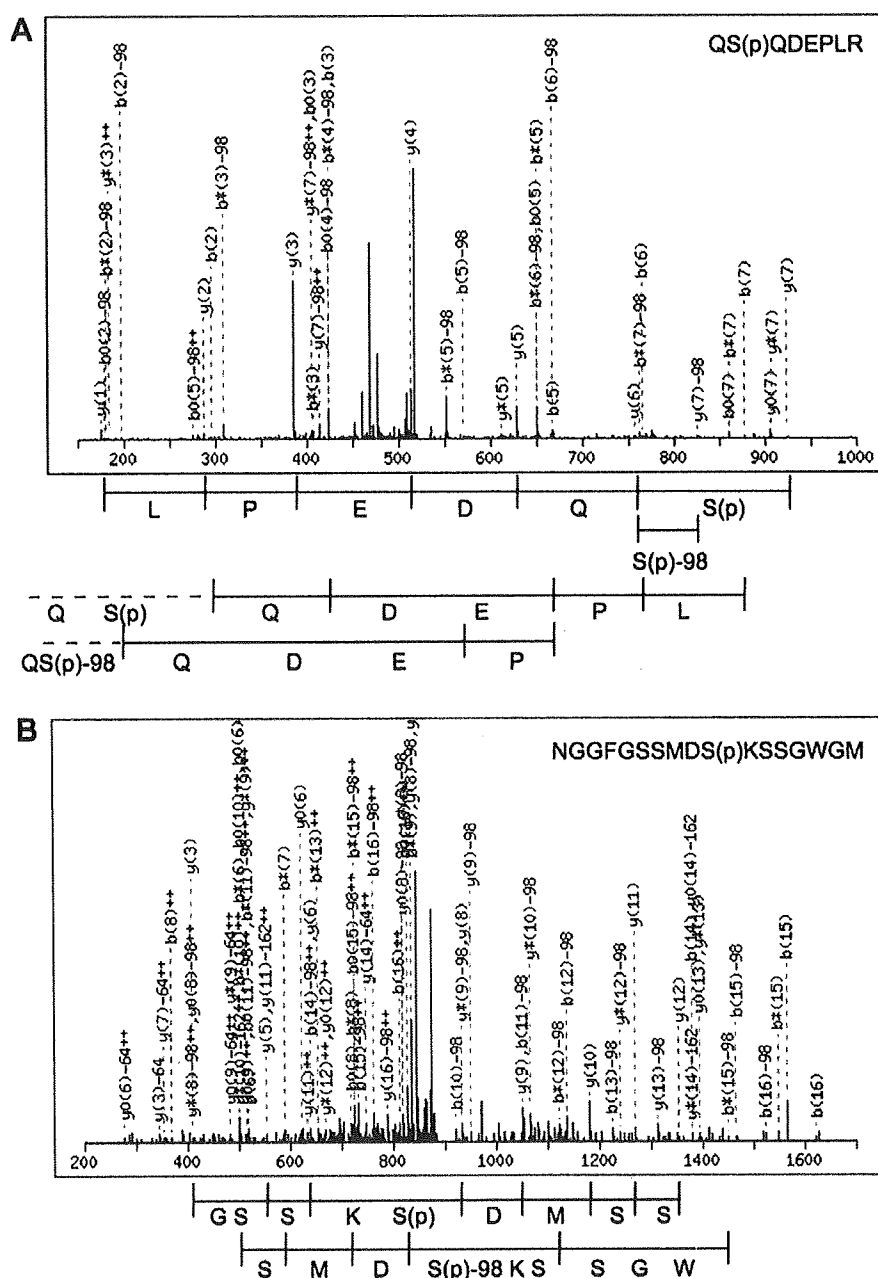


Fig. 1. Identification of phosphorylation sites by LC-MS/MS analysis. (A) Tryptic peptide, QSQDEPLR. The second Ser residue was phosphorylated. (B) Chymotryptic peptide, NGGFGSSMDSKSSGWGM. The eleventh Ser residue was phosphorylated.

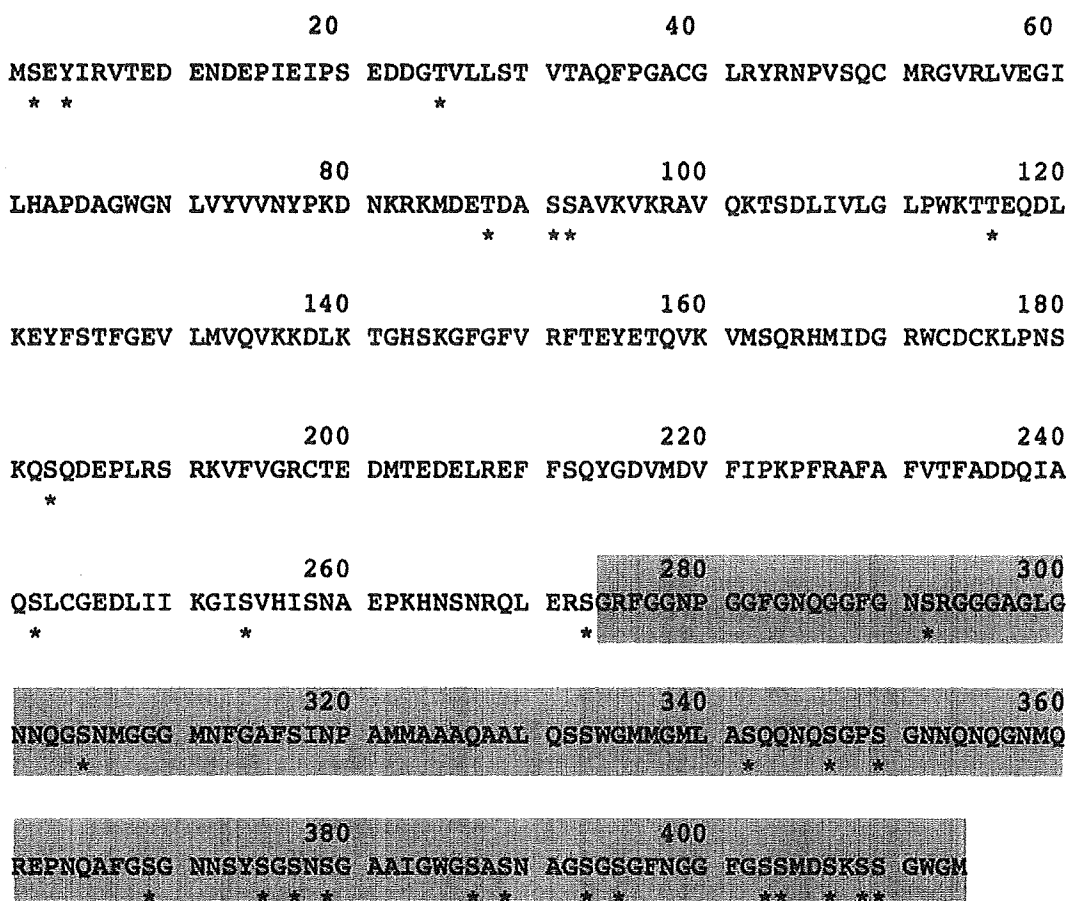


Fig. 2. CK1 phosphorylation sites on recombinant TDP-43. Asterisks show phosphorylation sites. The boxed region is the C-terminal Gly-rich region (273-414).

Table 2

The summarized table of prediction scores of CK1 phosphorylation and actual phosphorylated residues (number of phosphorylated residues/number of Ser/Thr residues).

Prediction score range	1-273	274-414 (Gly-rich region)
0.50-0.51	0	1/1
0.40-0.49	4/7	5/7
0.36-0.39	6/27	12/14

## Discussion

In this experiment, we identified 29 phosphorylation sites in recombinant TDP-43, including the previously reported Ser 91 and Ser 92 [11]. In particular, 18 of them were located in the C-terminal Gly-rich region. This suggests that the C-terminal Gly-rich region has a conformation that is favorable for CK1 phosphorylation. Interestingly, among 13 pathogenic mutations (G290A, G294V, G298S, A315T, S332N, M337V, Q343R, N345K, G348C, N352S, S379P, A382T, and I383V) found in familial ALS cases and 15 variants (D169G, N267S, G287S, G294A, G295S, G295R, Q331K, G335D, G348C, R361S, S379C, A382T, N390D, N390S, and S393L) found in single sporadic ALS cases [14-21], most are located in the C-terminal Gly-rich region.

The C-terminal Gly-rich region of TDP-43 regulates the alternative splicing of several genes [22,23] and functions as a transcriptional repressor [24]. This domain also binds several heterogeneous nuclear ribonucleoproteins [2,25]. Furthermore, using a yeast TDP-43 proteinopathy model, it was shown that the C-terminal Gly-rich region is required for TDP-43 aggregation and cellular toxicity in vivo [26].

Therefore, phosphorylation and/or mutation of amino acid residue(s) in the C-terminal Gly-rich region of TDP-43 may significantly affect cellular function.

Previously, we reported that recombinant TDP-43 hyperphosphorylated with CK1 showed similar electrophoretic mobility to abnormally phosphorylated TDP-43 observed in the brains of patients with FTLD-U and ALS, and we suggested that CK1 was involved in the abnormal phosphorylation of TDP-43 [7,8]. We prepared 39 antibodies to predicted phosphorylation sites in TDP-43 [7]. Twenty-three of these predicted sites were found to be actually phosphorylated in vitro by CK1. Furthermore, 10 antibodies against actual phosphorylated sites, pS91, pS379, pS389, pS395, pS403, pS404, p403/404, pS409, p410 and pS409/410, were found to label hyperphosphorylated recombinant TDP-43, not non-phosphorylated recombinant TDP-43 [7,8, unpublished data]. Therefore, the specificity of these antibodies was confirmed.

Antibodies raised against Ser 379, Ser 403/404 and Ser 409/410 sites in the C-terminal Gly-rich region labeled TDP-43 accumulations and/or inclusion bodies in brains of patients with FTLD-U and ALS; they also recognized abnormally phosphorylated TDP-43 in the Sarkosyl-insoluble fractions from FTLD-U and ALS brains, and labeled abnormal fibers of 15 nm diameter in inclusion bodies in the spinal cord of ALS patients [7,8]. Furthermore, these antibodies recognized high-molecular-weight smearing, abnormally phosphorylated TDP-43 with a molecular weight of 45 kDa, and C-terminal fragments with molecular weights of 18-28 kDa in Sarkosyl-insoluble fractions from FTLD-U and ALS brain [7,8]. Therefore, at least the Ser 379, Ser 403/404 and Ser 409/410 sites in the C-terminal Gly-rich region, which were identified as in vivo targets of CK1 phosphorylation, may be abnormally phosphorylated in

FTLD-U and ALS brains. Recently, rat monoclonal antibodies raised against phosphorylated Ser 409/410 gave similar results [27], supporting our previous findings. Thus, abnormal phosphorylation of the C-terminal Gly-rich region of TDP-43 is a consistent feature of pathologic inclusions in FTLD-U and ALS brains [7,8,27], and may play a significant role in the cytoplasmic accumulation and the formation of inclusion bodies, as in the cases of tauopathy and alpha-synucleinopathy [28,29].

In summary, our results suggest that multiple Ser residues in the C-terminal Gly-rich region of TDP-43 are major CK1 phosphorylation targets. Furthermore, phosphorylation of Ser 379, Ser 403/404 and Ser 409/410, which we observed previously in TDP-43 accumulated in FTLD-U and ALS brains [7], is also mediated by CK1, indicating that CK1 may be involved in pathological phosphorylation of TDP-43 in vivo. The data on phosphorylation sites of TDP-43 presented here should be useful for further studies on the mechanism of TDP-43 proteinopathy.

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## **A Cellular Model To Monitor Proteasome Dysfunction by $\alpha$ -Synuclein**

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## A Cellular Model To Monitor Proteasome Dysfunction by $\alpha$ -Synuclein<sup>†</sup>

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**ABSTRACT:** Impairment of the ubiquitin-proteasome degradation system has recently been suggested to be related to the onset of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. In this study, we investigated whether intracellular  $\alpha$ -synuclein affects proteasome activity in SH-SY5Y cells. To monitor intracellular proteasome activity, we used a reporter consisting of a short peptide degron fused to the carboxyl-terminus of green fluorescent protein (GFP-CL1), which is known to be degraded by proteasome. The level of intact GFP-CL1 was dramatically increased by coexpression of GFP-CL1 and  $\alpha$ -synuclein, as judged by confocal microscopic and immunoblot analyses. Expression of two pathogenic mutants of  $\alpha$ -synuclein, A30P and A53T, and phosphomimetic S129D mutant increased the intensities of GFP more effectively than did wild-type  $\alpha$ -synuclein. GFP fluorescence in cells transfected with  $\Delta$ 73-83 mutant or  $\beta$ -synuclein, which does not assemble into filaments *in vitro*, was not changed as compared with that in cells expressing GFP-CL1 alone. Thus, the ability of  $\alpha$ -synuclein to inhibit proteasome activity is related to its propensity to assemble into filaments. Furthermore, we observed that some compounds inhibiting  $\alpha$ -synuclein filament formation *in vitro* prevented the  $\alpha$ -synuclein-mediated proteasome dysfunction in cells transfected with both GFP-CL1 and  $\alpha$ -synuclein. The cellular model expressing both GFP-CL1 and  $\alpha$ -synuclein may be a useful tool to screen compounds protecting neurons from  $\alpha$ -synuclein-mediated proteasome dysfunction.

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. It is characterized by loss of dopaminergic neurons in the substantia nigra and by intracellular inclusion bodies known as Lewy bodies and Lewy neuritis.  $\alpha$ -Synuclein is a natively unfolded protein and is deposited in a hyperphosphorylated form as the major component of these filamentous inclusions (1–3). Genetic studies have revealed that missense mutations (A30P, E46K, and A53T) in the  $\alpha$ -synuclein gene cause familial forms of PD and dementia with Lewy bodies (DLB) (4–6). It has also been reported that duplications (duplication and triplication) of  $\alpha$ -synuclein gene cause an inherited form of PD and DLB (7–11). Thus, an increased level of intracellular  $\alpha$ -synuclein is thought to contribute to the onset of familial PD.

Intracellular and extracellular protein deposits may cause neuronal cell death and may signal onset of many neurodegenerative diseases. We have reported that phosphorylated  $\alpha$ -synuclein is partially ubiquitinated in  $\alpha$ -synucleinopathy brains (12). We also found that ubiquitination of  $\alpha$ -synuclein occurs in cultured cells at sites identical to those of filamentous  $\alpha$ -synuclein ubiquitinated *in vitro*, suggesting that ubiquitination may be a late event in the formation of Lewy bodies (13). A recent paper confirmed our findings and showed that monoubiquitination and multiubiquitination at Lys12, 21, and 23 are detectable

on phosphorylated  $\alpha$ -synuclein in  $\alpha$ -synucleinopathy brains (14). These findings indicate that the ubiquitin-proteasome system (UPS) is targeted to abnormal  $\alpha$ -synuclein in the brains of patients but that ubiquitinated  $\alpha$ -synuclein cannot be degraded by the UPS, suggesting that the UPS may not be effective to clear the abnormal form of  $\alpha$ -synuclein.

The UPS functions in cellular quality control by degrading misfolded, unassembled, or damaged proteins that might create toxic intracellular aggregates (15, 16). As the proteasome efficiently degrades multiubiquitinated proteins, the presence of elevated levels of ubiquitin conjugates in intracellular deposits of aggregated proteins in most neurodegenerative diseases suggests a linkage between UPS dysfunction and pathogenesis. Functional relationships between  $\alpha$ -synuclein and the UPS have been described (17–19). Synder et al. reported that aggregated and monomeric  $\alpha$ -synuclein inhibit proteasomal function *in vitro* (20). Lindersson et al. also observed proteasomal inhibition by  $\alpha$ -synuclein filaments and oligomers *in vitro* (21). In cultured cells, it remains controversial whether expression of  $\alpha$ -synuclein affects intracellular proteasome activity. Martin-Clemente et al. reported that  $\alpha$ -synuclein expression level does not significantly affect proteasome function in PC12 cells (22), while Fujita et al. reported that proteasome activity was significantly decreased in cells overexpressing wild-type  $\alpha$ -synuclein (23). Stefanis et al. described that expression of A53T mutant, but not wild-type  $\alpha$ -synuclein, inhibits proteasome activity in PC12 cells (24). It was also reported that cell-produced  $\alpha$ -synuclein oligomers are targeted to and impair the 26S proteasome (25).

Recently, Bence et al. reported impairment of the ubiquitin-proteasome system by the expression of an exon 1 fragment of

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huntingtin containing expanded polyglutamine homopolymer in cultured cells (26). They used a green fluorescent protein (GFP) reporter that is specifically degraded by the cellular proteasome. This fluorescent reporter system is also useful for facile detection of proteasome activity in *Caenorhabditis elegans* (27).

In this study, to investigate whether intracellular  $\alpha$ -synuclein affects proteasome activity, we have established a more sensitive assay system to monitor proteasome activity in cells using the GFP reporter and demonstrated impairment of proteasome function by expression of  $\alpha$ -synuclein in SH-SY5Y cells. We also found that two pathogenic mutations of  $\alpha$ -synuclein (A30P and A53T) and S129D mutant, which mimics phosphorylation, inhibit proteasome activity more effectively than does wild-type  $\alpha$ -synuclein, while  $\Delta$ 73-83 mutant and  $\beta$ -synuclein, which do not form fibrils *in vitro*, do not affect proteasome function. Furthermore, we used our cellular model to screen for compounds protecting cells from proteasome inhibition by  $\alpha$ -synuclein and found that some polyphenols attenuate  $\alpha$ -synuclein-induced proteasome impairment.

## EXPERIMENTAL PROCEDURES

**Construction of Plasmids.** The GFP-CL1 plasmid was constructed by digestion of pEGFP-C1 (Clontech) with *Bgl*III and *Sall* and ligation with hybridized oligonucleotides termed CL1s (5'-GATCCGCTTGTA AAAATTGGTTTTCTTCTTT-ATCTCATTGTTATTTCATTATAAG-3') and CL1a (5'-TCGACTTATAAATGAATAACAAAATGAGATAAAGAAGAAAACCAATTTTACAAGCG-3'). The resulting construct, the GFP gene containing an oligonucleotide encoding ACKNWFSSLSHFVIHL, was confirmed by DNA sequencing and designated pEGFP-CL1.

Human  $\alpha$ -synuclein,  $\beta$ -synuclein, tau 3R1N, and 4R1N cDNAs in pRK172 vector were kind gifts from Dr. M. Goedert. The open reading frames of  $\alpha$ -synuclein,  $\beta$ -synuclein, tau 3R1N, and 4R1N were subcloned into the mammalian expression vector pcDNA3. To construct plasmids encoding  $\alpha$ -synuclein mutants A30P, A53T, S129A, and S129D, we performed site-directed mutagenesis using a site-directed mutagenesis kit (Stratagene). We constructed a deletion mutant (residues 73–83:  $\Delta$ 73-83) of  $\alpha$ -synuclein using PCR with the forward primer (5'-GGAGCAGGGAGCATTGCAGCA-3') and the reverse primer (5'-CGTCCACTGCTCCTCCAAC-3') using pcDNA3- $\alpha$ -synuclein as a template. The amplified fragments were self-ligated, and the resulting plasmids designated pcDNA- $\alpha$ -synuclein  $\Delta$ 73-83. All constructs were verified by DNA sequencing.

**Cell Culture and Plasmid Expression.** SH-SY5Y cells were cultured in DMEM/F12 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, Penicillin-Streptomycin-Glutamine (GIBCO), and MEM Non-Essential Amino Acids Solution (GIBCO). The cells were maintained at 37 °C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub>. Cells were grown to 50% confluence in six-well culture dishes for transient expression and then transfected with various expression plasmids using FuGENE6 (Roche) according to the manufacturer's instructions.

**Confocal Immunofluorescence Microscopy.** SH-SY5Y cells were grown on a coverslip (15 mm  $\times$  15 mm) and transfected with expression vector (total 1.3  $\mu$ g, e.g., 1  $\mu$ g of pcDNA3- $\alpha$ -synuclein plus 0.3  $\mu$ g of pEGFP-CL1). After incubation for indicated times, transfected cells on the coverslips were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. The coverslips were then incubated in 50 mM NH<sub>4</sub>Cl

in PBS for 10 min, and cell permeabilization was performed with 0.2% (v/v) Triton X-100 in PBS for 10 min. The cells were blocked for 30 min in 5% (w/v) BSA in PBS, then incubated with anti- $\alpha$ -synuclein monoclonal antibody, Syn102 (28) (1:500 dilution) for 1 h at 37 °C, followed by TRITC-labeled goat anti-mouse IgG (Sigma, 1:500 dilution) as the secondary antibody for 1 h at 37 °C. They were washed again and further incubated with TO-PRO-3 (Molecular Probes, 1:3000 dilution in PBS) for 45 min at 37 °C to stain nuclear DNA and analyzed using a LSM5 Pascal confocal laser microscope (Zeiss).

**Sequential Extraction of Proteins and Immunoblotting.** SH-SY5Y cells were grown in a six-well plate and transiently transfected with expression plasmids (total 1.3  $\mu$ g). After incubation for 48–72 h, cells were harvested and lysed in TS buffer [50 mM Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 5 mM ethylenediaminetetraacetic acid, 5 mM ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N,N*-tetraacetic acid, and protease inhibitor cocktail (Roche)]. The lysate was centrifuged at 290,000g for 20 min at 4 °C, and the supernatant recovered as the TS-soluble fraction. The TS-insoluble pellet was lysed in TS buffer containing 1% (v/v) Triton X-100 (TX) and centrifuged at 290,000g for 20 min at 4 °C. The supernatant was collected as the TX-soluble fraction. The TX-insoluble pellet was further lysed in SDS-sample buffer and heated for 5 min. Protein concentration was estimated by using the BCA Protein Assay Kit (Pierce). Samples (20  $\mu$ g) were separated by 13.5% (v/v) SDS-PAGE using the Tris-tricine buffer system and proteins were transferred onto polyvinylidene difluoride membrane (Millipore). The blots were then blocked with 3% (v/v) gelatin and incubated overnight with anti-GFP monoclonal antibody (MBL), anti-Syn102, anti-human tau monoclonal antibody (HT7, Thermo Scientific) or anti- $\alpha$ -tubulin monoclonal antibody (Sigma) in 10% (v/v) calf serum at a dilution of 1:1,000–5,000 at room temperature. After washing, blots were incubated with horseradish peroxidase-labeled secondary antibody (Bio-Rad) at a dilution of 1:10,000 in 10% (v/v) calf serum for 1 h at room temperature or a biotin-labeled secondary antibody (Vector) for 2 h at room temperature. Signals were detected using the chemiluminescence reagent Immunostar (Wako) or the ABC staining kit (Vector).

**Measurement of Proteasome Activity Using a Peptide Substrate.** SH-SY5Y cells transfected with expression plasmids were cultured for 72 h or treated with 20  $\mu$ M MG132 for 6 h. Cells were harvested and cytosolic fraction was prepared as follows. Cells were resuspended in 100  $\mu$ L of phosphate-buffered saline (PBS) and disrupted by sonication. Insoluble material was removed by ultracentrifugation at 290,000g for 20 min at 4 °C. The supernatant was assayed for proteasome activity by using a fluorescent peptide substrate, benzyloxycarbonyl-Leu-Leu-Glu-7-amido-4-methylcoumarin (*z*-LLE-MCA, Peptide Institute, Inc.). Aliquots (10  $\mu$ L) were mixed with 0.1 M Tris-HCl buffer, pH 7.5 and water in a total volume of 1 mL. After preincubation at 37 °C for 10 min, 10  $\mu$ L of 10 mM peptide substrate was added and the mixture was incubated for 30 min. A solution of 2% (v/v) acetic acid (1 mL) was added to the reaction mixture, and 7-amino-4-methylcoumarin (AMC) release was measured fluorometrically (excitation wavelength of 365 nm; emission wavelength of 460 nm). Enzyme activity was described as arbitrary unit/mg protein.

**Screening for Compounds Preventing Proteasome Inhibition by  $\alpha$ -Synuclein in Cultured Cells.** SH-SY5Y cells were grown in six-well plates or on coverslips and transfected with both  $\alpha$ -synuclein (1  $\mu$ g) and GFP-CL1 plasmids (0.3  $\mu$ g).

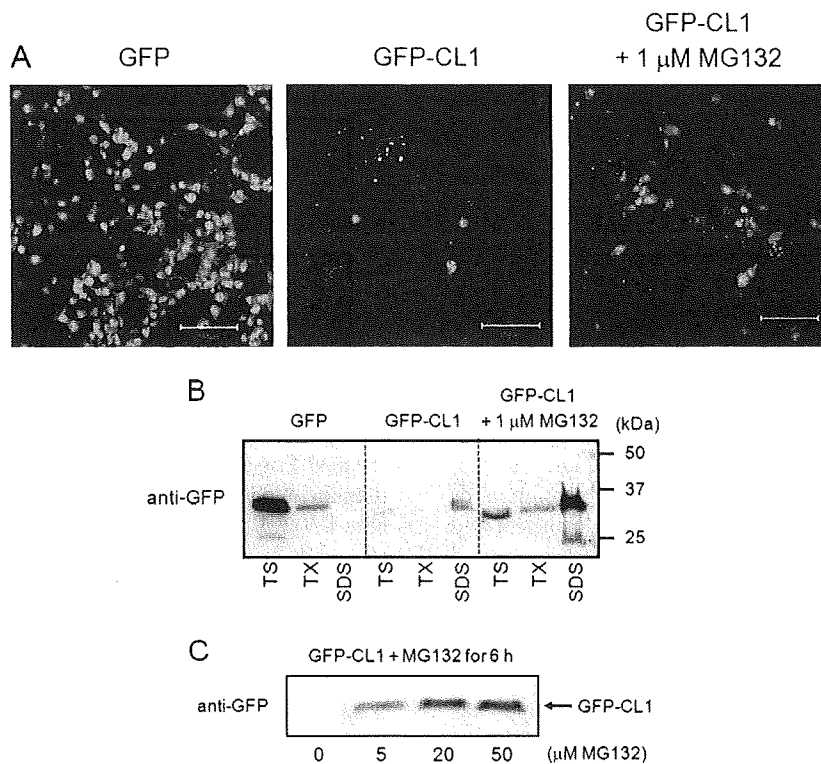


FIGURE 1: GFP-CL1 is degraded by proteasome in SH-SY5Y cells. (A) SH-SY5Y cells grown on coverslips were transfected with GFP or GFP-CL1 using FuGENE6. After an overnight incubation with or without  $1 \mu\text{M}$  MG132, cells were fixed (total 48 h after transfection), stained with TO-PRO-3 (Blue), and analyzed by confocal microscopy. Scale bar =  $100 \mu\text{m}$ . (B) SH-SY5Y cells were transfected with GFP or GFP-CL1 and treated overnight with or without  $1 \mu\text{M}$  MG132. Cells were harvested, and cell lysate was fractionated as described in Experimental Procedures and analyzed by immunoblotting using anti-GFP antibody. TS, Tris-soluble fraction; TX, Triton X-100-soluble fraction; SDS, SDS-soluble fraction. (C) SH-SY5Y cells were transfected with GFP-CL1 and treated with MG132 ( $0$ – $50 \mu\text{M}$ ) for 6 h. The cells were harvested, and the TX-insoluble fraction was analyzed by immunoblotting with anti-GFP antibody.

Various compounds (all at  $40 \mu\text{M}$ ) were added to the culture medium 2 h after transfection. Cells were harvested after incubation for 72 h, and the immunoblot and/or confocal microscopic analyses were performed as above. A decrease in GFP level in transfected cells treated with a compound, compared with the GFP level in transfected cells without such treatment, indicated that the compound rescues cells from proteasome dysfunction caused by expression of  $\alpha$ -synuclein.

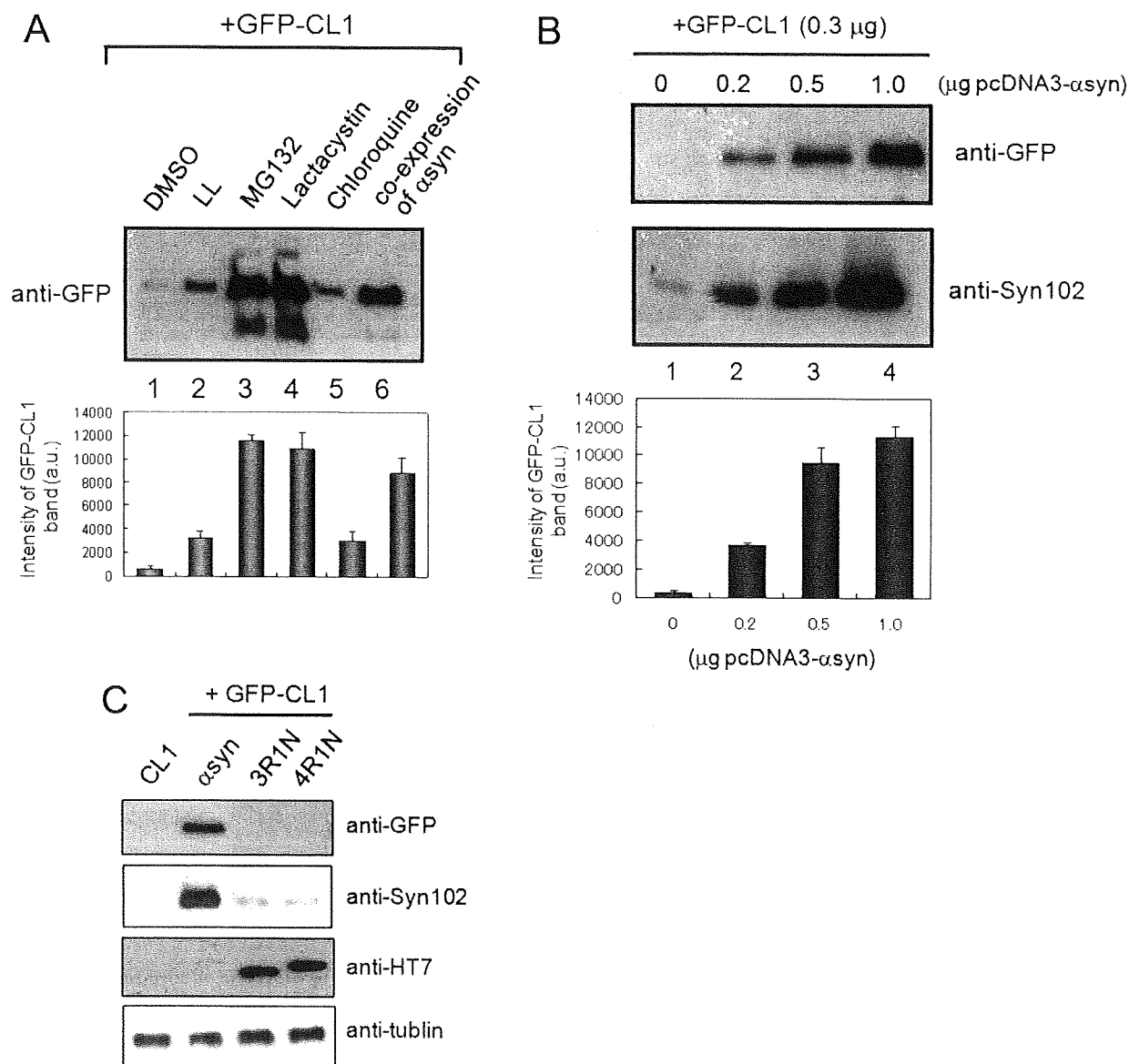
## RESULTS

**Cellular Expression of GFP-CL1.** To establish a sensitive and convenient assay system for proteasome activity in cultured cells, we constructed GFP-CL1, which is GFP fused at the C-terminus with the 16-residue degron peptide CL1 (26, 29). We transiently transfected SH-SY5Y cells with GFP or GFP-CL1, and analyzed the cells using confocal microscopy. As shown in Figure 1A, the fluorescence of GFP in cells transfected with GFP-CL1 was less than that in cells expressing GFP. The proteasome inhibitor MG132 increased fluorescence intensity in GFP-CL1-transfected cells (Figure 1A). Thus, we reconfirmed that the CL1 sequence specifically targeted normally stable GFP for efficient degradation by proteasome, as reported previously (26).

To biochemically characterize the proteasomal degradation of GFP-CL1, we fractionated lysates of cells transfected with GFP-CL1 or GFP. The TS-, TX-, and SDS-soluble fractions were analyzed by immunoblotting using anti-GFP antibody. As shown in Figure 1B, almost all GFP was recovered in the TS fraction in cells transfected with GFP, although a little GFP was

detected in the TX-soluble fraction. In contrast, in cells expressing GFP-CL1, only weak GFP immunoreactivity was detected in the TS- and TX-insoluble fractions because of GFP-CL1 degradation by proteasome in transfected cells. Interestingly, in cells expressing GFP-CL1 treated with MG132, a band of uncleaved GFP-CL1 was detected in the SDS-soluble fraction in addition to the weak bands seen in the TS- and TX-soluble fractions, suggesting that uncleaved GFP-CL1 unexpectedly aggregates in cells when proteasome activity is inhibited by MG132 treatment. We also reconfirmed that MG132 treatment effectively and dose-dependently blocked the degradation of GFP-CL1 (Figure 1C).

**Expression of  $\alpha$ -Synuclein Inhibits Cellular Proteasome Activity.** To investigate whether expression of  $\alpha$ -synuclein might affect proteasome function in cultured cells, we cotransfected both  $\alpha$ -synuclein and GFP-CL1 into SH-SY5Y cells. To reconfirm that GFP-CL1 is effectively degraded by proteasome, cells transfected with GFP-CL1 were treated with several protease inhibitors. SDS-soluble fraction of cell lysate was subjected to immunoblot analysis using anti-GFP antibody. As shown in Figure 2A, increased levels of GFP-CL1 band were observed in cells coexpressing GFP-CL1 and  $\alpha$ -synuclein (lane 6), indicating that proteasome activity is inhibited effectively by expression of  $\alpha$ -synuclein. Similar results were obtained when we treated cells expressing GFP-CL1 with MG132 or lactacystin (lane 3 and 4). On the other hand, leucyl-leucinal (LL, a calpain inhibitor) or chloroquine (a lysosomal inhibitor) did not inhibit GFP-CL1 degradation (lanes 2 and 5). We then tested whether the proteasome inhibition by  $\alpha$ -synuclein is dose-dependent, i.e., dependent on the  $\alpha$ -synuclein expression level. SH-SY5Y cells were



**FIGURE 2:** Expression of  $\alpha$ -synuclein inhibits proteasome activity in SH-SY5Y cells. (A) SH-SY5Y cells transfected with GFP-CL1 were treated with 1  $\mu$ M LL, MG132, lactacystin, and chloroquine overnight. In the case of coexpression, cells were transfected with both GFP-CL1 and  $\alpha$ -synuclein using FuGENE6. The cells were harvested, and the Triton X-100-insoluble and SDS-soluble fractions were prepared and analyzed by immunoblotting using an anti-GFP antibody. The results of quantitative analysis of the GFP-CL1 bands, expressed as means  $\pm$  SD ( $n = 3$ ), are shown below the blot. (B) SH-SY5Y cells were transfected with GFP-CL1 (0.3  $\mu$ g),  $\alpha$ -synuclein (0, 0.2, 0.5, or 1  $\mu$ g), and/or pcDNA3 empty vector (total plasmids = 1.3  $\mu$ g) and incubated for 72 h. The cells were harvested, and then Tris-soluble (lower panels) and SDS-soluble fractions (upper panels) were prepared. Immunoblot analyses were performed using anti-GFP (upper panels) or anti-Syn102 (lower panels) antibodies. The results of quantitative analysis of the GFP-CL1 bands, expressed as means  $\pm$  SD ( $n = 3$ ), are shown below the blot. (C) SH-SY5Y cells were transfected with both GFP-CL1 (0.3  $\mu$ g) and  $\alpha$ -synuclein (1  $\mu$ g) or pcDNA3-tau 3R1N or 4R1N vector (1  $\mu$ g) and incubated for 72 h. The cells were harvested, and the TX-insoluble fraction was analyzed by immunoblotting with anti-GFP antibody. The TS-soluble fraction was also analyzed by using anti-Syn102, anti-HT7, and anti-tubulin antibodies (as a loading control).

transfected with both pEGFP-CL1 (0.3  $\mu$ g) and pcDNA3- $\alpha$ -synuclein (0, 0.2, 0.5, or 1  $\mu$ g; total 1.3  $\mu$ g plasmids), followed by immunoblot analysis. The result showed that the level of intact GFP-CL1 increased in parallel with increased expression of  $\alpha$ -synuclein (Figure 2B). Furthermore, we tested whether expression of tau protein which is also an effective substrate for proteasome (30), inhibits intracellular proteasome activity in SH-SY5Y cells. We found that expression of tau protein (3R1N and 4R1N) did not affect the proteasome activity in SH-SY5Y cells, suggesting that the effect of  $\alpha$ -synuclein on the proteasome activity observed in this study is not simply due to overexpression of a protein, but is specific for  $\alpha$ -synuclein. We also confirmed the absence of significant cell death in our

cellular model transiently expressing  $\alpha$ -synuclein, suggesting that the decreased proteasome activity found in cells expressing  $\alpha$ -synuclein is not caused by cellular damage due to the toxicity of expressed  $\alpha$ -synuclein.

To visually monitor proteasome inhibition due to expression of  $\alpha$ -synuclein, SH-SY5Y cells were transiently cotransfected with  $\alpha$ -synuclein and GFP-CL1, and transfected cells were analyzed by confocal microscopy. As shown in Figure 3C, the numbers of GFP-positive cells were significantly increased in cells transfected with wild-type  $\alpha$ -synuclein and GFP-CL1, compared with cells transfected with GFP-CL1 alone (Figure 3B). This is in good agreement with the above immunoblotting data (Figure 2), indicating that expression of  $\alpha$ -synuclein inhibited proteasome activity.

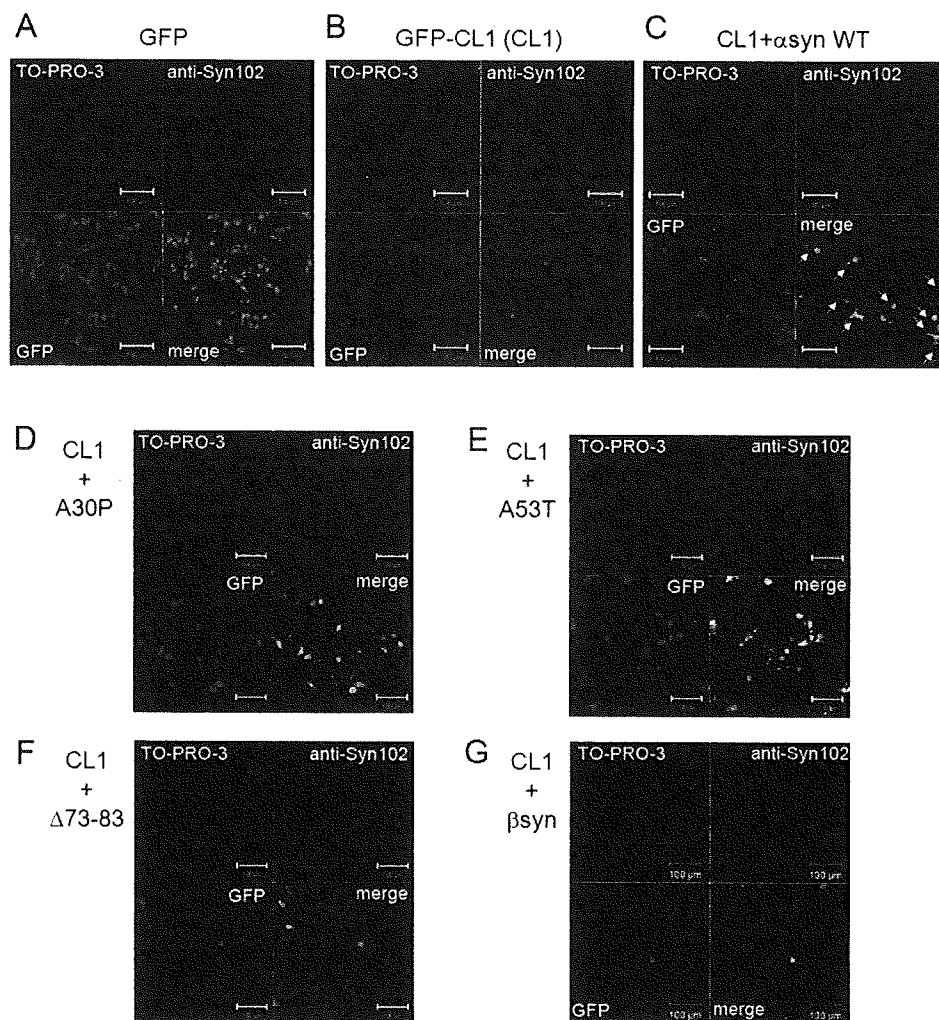


FIGURE 3: Expression of  $\alpha$ -synuclein mutants affects proteasome activity in SH-SY5Y cells. SH-SY5Y cells grown on coverslips were transfected with GFP (A), GFP-CL1 (B), or both GFP-CL1 and wild-type  $\alpha$ -synuclein (C), A30P (D), A53T (E),  $\Delta$ 73-83 (F), or  $\beta$ -synuclein (G). Cells were fixed 72 h after transfection and stained with anti-Syn102 (Red) and TO-PRO-3 (blue). Intracellular  $\alpha$ -synuclein inhibits proteasome activity in cells expressing both GFP-CL1 and  $\alpha$ -synuclein (arrows). Scale bar = 100  $\mu$ m.

Taken together, these results showed that proteasome activity can be specifically and sensitively analyzed by monitoring the fluorescence intensity or immunoreactivity of GFP-CL1 in cultured cells and that the expression of  $\alpha$ -synuclein impairs cellular proteasome function.

*Effects of Expression of  $\alpha$ -Synuclein Mutants and  $\beta$ -Synuclein on Proteasome Activity.* Recombinant  $\alpha$ -synuclein with a pathogenic mutation (A30P, A53T, or E46K) is more prone to assemble into filaments *in vitro* than the wild-type protein (31, 32).  $\beta$ -Synuclein and  $\gamma$ -synuclein (33) and the  $\alpha$ -synuclein deletion mutant  $\Delta$ 73-83 (deletion of 73–83 residues) did not form fibrils *in vitro* (Nonaka et al., unpublished data). To study the effects of  $\alpha$ -synuclein mutations on cellular proteasome activity, we transiently cotransfected these mutants and GFP-CL1 into SH-SY5Y cells and examined the cells using confocal microscopy. As shown in Figure 3, we found that increased GFP signals were observed in cells transfected with A30P (Figure 3D) or A53T (Figure 3E) compared with the signal seen in cells transfected with wild-type  $\alpha$ -synuclein (Figure 3C), although the expression levels of the  $\alpha$ -synuclein proteins were almost equal (Figure 3 and 4A). In contrast, little GFP fluorescence was detected in cells transfected with the  $\alpha$ -synuclein deletion mutant  $\Delta$ 73-83 (Figure 3F) or  $\beta$ -synuclein (Figure 3G).

We also quantitatively examined the effects of these  $\alpha$ -synuclein mutations on proteasome activity using immunoblot analysis. SH-SY5Y cells were transfected with both GFP-CL1 and wild-type  $\alpha$ -synuclein, or the A30P, A53T, or  $\Delta$ 73-83 mutant, or  $\beta$ -synuclein. Uncleaved GFP-CL1 was recovered in the TX-insoluble (SDS-soluble) fraction as described above. As shown in Figure 4A, the level of uncleaved GFP-CL1 in cells transfected with A53T was about 30% higher than that in cells transfected with wild-type  $\alpha$ -synuclein (Figure 4A, lane 4). A slightly higher level of GFP-CL1 was detected in cells transfected with the A30P mutant (Figure 4A, lane 3) compared with that in cells transfected with wild-type  $\alpha$ -synuclein (Figure 4A, lane 2). In contrast, the level of GFP-CL1 in cells transfected with  $\Delta$ 73-83 or  $\beta$ -synuclein was negligible, as was that in control cells (Figure 4A, lane 5 and 6). The expression levels of  $\alpha$ -synuclein protein in Tris-soluble fractions were almost the same. These results showed that mutants of  $\alpha$ -synuclein that are prone to aggregate *in vitro* have stronger inhibitory effects on cellular proteasome activity than does the wild-type protein and that  $\Delta$ 73-83 and  $\beta$ -synuclein, neither of which form fibrils *in vitro*, do not inhibit the proteasome.

*Expression of Phosphomimetic  $\alpha$ -Synuclein Mutant S129D Enhanced Its Inhibition of Proteasome Activity.* Aggregated  $\alpha$ -synuclein in brains of  $\alpha$ -synucleinopathy patients

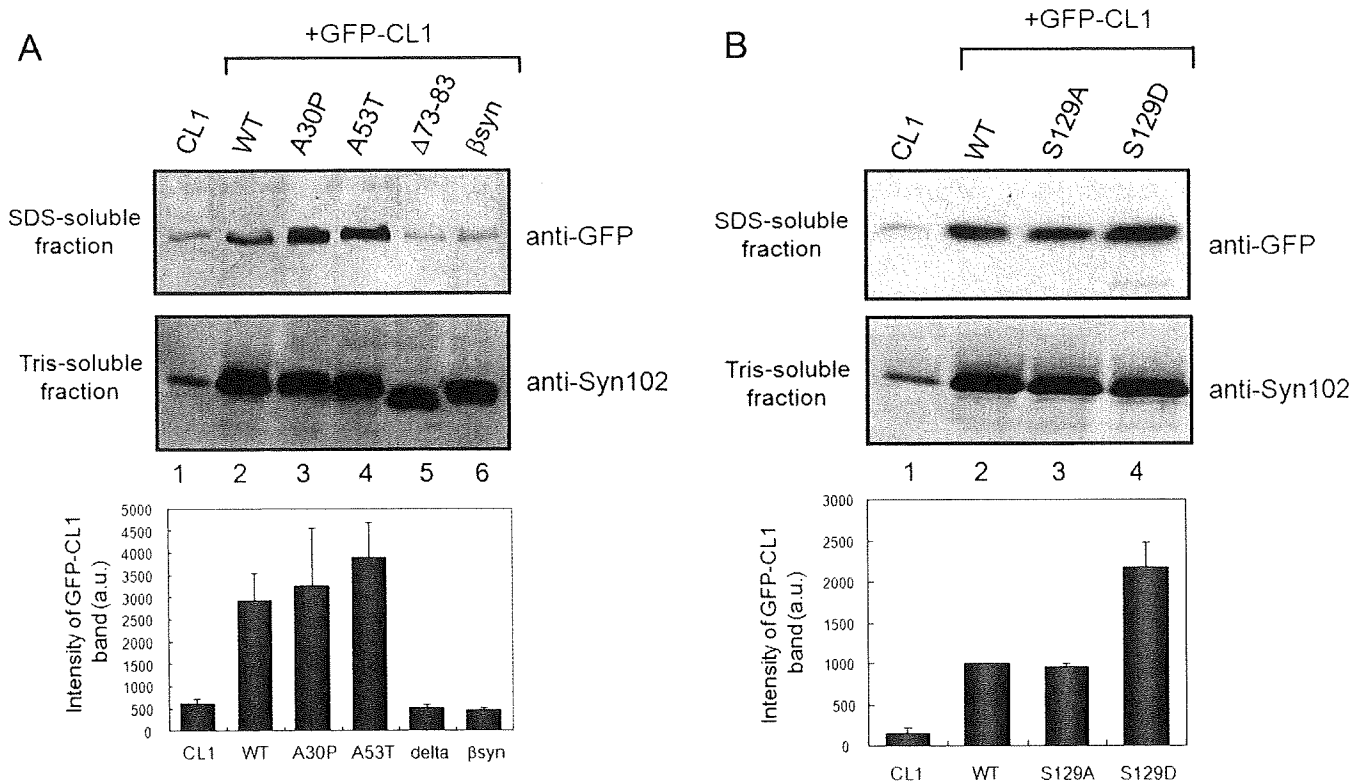


FIGURE 4: Effects of expression of  $\alpha$ -synuclein mutants on proteasome activity. (A, B) Immunoblot analyses of extracted proteins from cells transfected with both GFP-CL1 and wild-type  $\alpha$ -synuclein or a mutant (A30P, A53T, S129A, S129D,  $\Delta$ 73-83 or  $\beta$ -synuclein). Cells were harvested 72 h after transfection, and Tris-soluble (lower panels) and SDS-soluble fractions (upper panels) were prepared. Immunoblot analyses were performed using anti-GFP (upper panels) or anti-Syn102 (lower panels) antibodies. The results of quantitative analysis of the GFP-CL1 bands, expressed as means + SD ( $n = 3$ ), are shown below the blot.

is hyperphosphorylated at Ser129, and this post-translational modification facilitates self-aggregation *in vitro* (3). Altering Ser129 to the negatively charged residue Asp (S129D) to mimic phosphorylation significantly enhances  $\alpha$ -synuclein toxicity in a *Drosophila* model (34). We asked whether phosphorylation of  $\alpha$ -synuclein might affect proteasome activity in cultured cells. SH-SY5Y cells were transfected with GFP-CL1 and wild-type or mutant  $\alpha$ -synuclein. Immunoreactivity of GFP-CL1 in cells transfected with S129A was similar to that of cells transfected with wild-type  $\alpha$ -synuclein, while the levels of GFP-CL1 in cells expressing S129D mutant to mimic phosphorylation was significantly higher than those in cells transfected with the wild-type or S129A (Figure 4B). These results suggested that phosphorylation of Ser129 may facilitate inhibition of proteasome function by intracellular  $\alpha$ -synuclein.

**Comparison of Proteasome Activity Assay Using GFP-CL1 with a Conventional Cleaving Assay Using a Fluorescent Peptide Substrate.** To compare the sensitivity of the assay of proteasome activity using GFP-CL1 with that of the conventional assay using a fluorescent peptide, we evaluated biochemically the proteasome activity of lysates from cells transfected with  $\alpha$ -synuclein. SH-SY5Y cells were transfected with wild-type  $\alpha$ -synuclein for 72 h, and proteasome activity in cell lysates was measured using a peptide substrate specific for proteasome, zLLE-MCA. As shown in Figure 5, zLLE-MCA-cleaving activity in cells transfected with wild-type  $\alpha$ -synuclein was decreased slightly (~10% inhibition) compared with that in cells transfected with empty vector, while MG132 had a potent inhibitory effect on proteasome activity (~80% inhibition). On the other hand, as described above, the levels of GFP-CL1 in cells transfected with GFP-CL1 and  $\alpha$ -synuclein were increased about

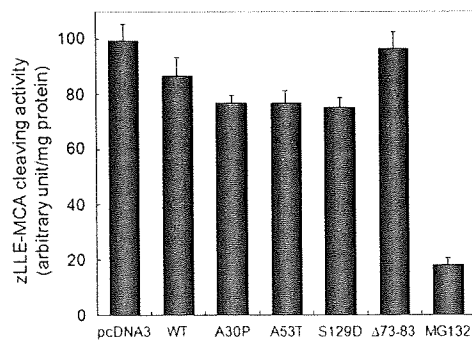


FIGURE 5: Measurements of proteasome activity using a fluorescent peptide substrate. SH-SY5Y cells were transfected with pcDNA3 empty vector, wild-type  $\alpha$ -synuclein (WT), A30P, A53T, S129D,  $\Delta$ 73-83 for 72 h or treated with 20  $\mu$ M MG132 for 6 h. Cytosolic fractions were prepared and assayed using zLLE-MCA as a substrate. The results were expressed as means + SD ( $n = 3$ ).

5 times compared with those in cells expressing GFP-CL1 (Figure 4A).

As shown in Figure 5, the zLLE-MCA-cleaving activity in cells transfected with A30P was almost equal to that in cells expressing A53T, which in turn was decreased slightly compared with that in cells transfected with wild-type  $\alpha$ -synuclein. Proteasome activity in cells transfected with  $\Delta$ 73-83 was found to be the same as that in cells transfected with empty vector. It was also revealed that S129D had a slightly stronger inhibitory effect on proteasome activity than did wild-type  $\alpha$ -synuclein. Taken together, as compared with the results shown in Figure 3 and 4, these results clearly indicate that a method in this study by detecting the fluorescence of GFP-CL1 or immunoblot analysis using



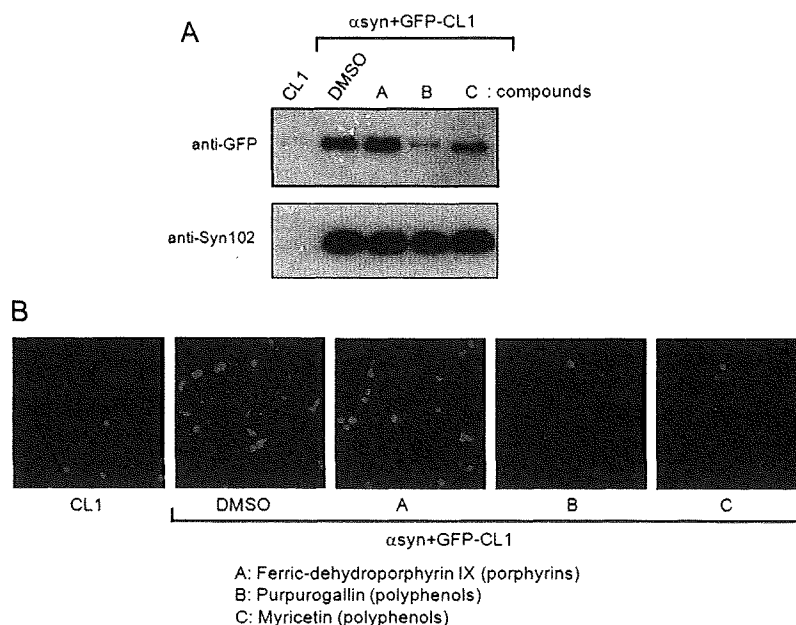


FIGURE 6: Small molecules prevent proteasome dysfunction by intracellular  $\alpha$ -synuclein. SH-SY5Y cells were transfected with GFP-CL1 alone or both GFP-CL1 and  $\alpha$ -synuclein. Compounds (final  $40 \mu\text{M}$ ) were added 2 h after transfection. Cells were harvested after a 72-h incubation and analyzed by immunoblotting (A) and confocal microscopy (B). Immunoblot analyses of Tris-soluble (lower panels) and SDS-soluble fractions (upper panels) were performed using anti-GFP (upper panels) or anti-Syn102 (lower panels) antibodies. The decreased GFP-CL1 level in transfected cells treated with compound B or C, as compared with that of transfected cells treated with vehicle alone (DMSO), indicated that compound B or C, but not compound A, rescued cells from proteasome impairment by expression of  $\alpha$ -synuclein.

anti-GFP antibody is a simple and sensitive for monitoring proteasome activity in cells.

*Small Molecular Compounds Attenuate Cellular Inhibition of Proteasome Activity by Expression of  $\alpha$ -Synuclein.* In this study, we have shown that proteasomal activity is impaired by expression of  $\alpha$ -synuclein in SH-SY5Y cells. Impairment of proteasome function leads to induction of apoptosis, followed by cell death, so it is important to protect cells from such inhibition by intracellular  $\alpha$ -synuclein. These results raised the possibility that our cellular model, in which both GFP-CL1 and  $\alpha$ -synuclein are expressed, might be useful to screen for small molecules preventing proteasome inhibition mediated by expression of  $\alpha$ -synuclein. Recently, we have reported that several compounds, including polyphenols, phenothiazines, and porphyrins, inhibit aggregation of recombinant  $\alpha$ -synuclein *in vitro* (35). We therefore asked whether these compounds might affect proteasome inhibition by  $\alpha$ -synuclein in cultured cells. Compounds of interest were added to the culture medium of cells transfected with both GFP-CL1 and  $\alpha$ -synuclein 2 h after transfection. The cells were incubated for 72 h, harvested and analyzed by immunoblotting and confocal laser microscopy. Immunoblot analyses showed that the level of GFP-CL1 was decreased significantly when cells were treated with purpurogallin or myricetin (polyphenols), but not when they were treated with ferric-dehydroporphyrin IX (porphyrin) (Figure 6A). Cells treated with purpurogallin or myricetin displayed significant decreases in GFP-CL1 fluorescence by confocal microscopy (Figure 6B). Furthermore, we confirmed that exifone and gossypetin, plant polyphenols inhibiting fibril formation of  $\alpha$ -synuclein *in vitro* (35), also attenuated  $\alpha$ -synuclein-mediated proteasome inhibition (data not shown). We further observed that these small molecules did not affect  $\alpha$ -synuclein expression (Figure 6A). These results indicate that these compounds may protect cells from proteasome dysfunction arising from expression of  $\alpha$ -synuclein.

## DISCUSSION

Recent studies have shown that aggregated and monomeric  $\alpha$ -synuclein inhibit proteasomal function *in vitro* (20, 21, 36). In these reports, proteasome activity was measured in a cell-free system using fluorescent peptide substrates, and little is known about the effects of  $\alpha$ -synuclein expression on proteasome activity in cultured cells. To examine this question, we tried to establish a sensitive and convenient method to measure proteasome activity using cultured cells transfected with a GFP-CL1 reporter plasmid. We further applied this system to show that transient coexpression of  $\alpha$ -synuclein inhibits the proteasome activity of SH-SY5Y cells.

The GFP-CL1 reporter is a useful tool for the detection of proteasome activity in cells (26). Recently, Link et al. reported that the expression of GFP-CL1 in *Caenorhabditis elegans* muscle or neurons causes deposition of GFP-CL1 and leads to rapid paralysis (27). In this study, we also found that uncleaved GFP-CL1 was largely recovered in TX-insoluble fractions of SH-SY5Y cells transfected with GFP-CL1 and  $\alpha$ -synuclein, while  $\alpha$ -synuclein was recovered in TS- and TX-soluble fractions (Figure 1B and Figure S1 in Supporting Information), indicating that uncleaved GFP-CL1 was deposited, but  $\alpha$ -synuclein was still soluble in transfected cells. Cell death was not detected in either cells transfected with GFP-CL1 alone or cells transfected with both GFP-CL1 and wild-type  $\alpha$ -synuclein or mutants thereof, although Link et al. found that GFP-CL1 is toxic when expressed in *C. elegans* muscle cells (27). The discrepancy might be due to differences in the cell type used. Further studies are required in this regard. It is surprising that a relatively short addition of 16 residues to the C terminus of GFP can convert this normally soluble protein into a protein that strongly aggregates. We suggest that the C-terminal 16 residues may directly interfere with GFP folding, resulting in misfolded (but still fluorescent) forms that are prone to aggregate.



How does  $\alpha$ -synuclein suppress intracellular proteasome activity? Several reports have described that  $\alpha$ -synuclein may directly interfere with the proteasome. It was shown that  $\alpha$ -synuclein binds to S6' or S6a, a component of the 19S subunit in the 26S proteasome in cultured cells (23, 37). It was also reported that aggregated  $\alpha$ -synuclein, but not its monomer, effectively inhibited the 26S proteasome activity *in vitro* (20, 21, 25, 36). Interestingly, we have shown that two pathogenic  $\alpha$ -synuclein mutants, A30P and A53T, which are liable to form fibrils or oligomers *in vitro*, inhibited proteasome activity more strongly than did wild-type  $\alpha$ -synuclein, while  $\Delta$ 73-83 mutant and  $\beta$ -synuclein, neither of which assemble into filaments *in vitro*, did not inhibit proteasome activity. Although we failed to detect any sarkosyl-insoluble aggregates of  $\alpha$ -synuclein in cells transfected with wild-type or mutant  $\alpha$ -synuclein (data not shown), these results raise the possibility that soluble prefibrillar intermediates, such as misfolded monomers, oligomers or protofibrils, may inhibit proteasome activity in transfected cells. Additional studies are needed to explore oligomers and protofibrils of  $\alpha$ -synuclein in our cell model.

Phosphorylation seems to be a common mechanism controlling the neurotoxicity of aggregation-prone toxic proteins involved in neurodegenerative diseases such as tau (38, 39),  $\alpha$ -synuclein (3), and TDP-43 (40–42). We suggested that phosphorylation of  $\alpha$ -synuclein may play a role in suppression of proteasome activity in cells. It was reported that expression of S129D mutant  $\alpha$ -synuclein in *Drosophila* significantly enhances the toxicity (34). However, the mechanisms through which phosphomimetic S129D mutant causes cell death in the fly remain unknown. Phosphorylated  $\alpha$ -synuclein at Ser129 is known to be more prone to aggregate than wild-type  $\alpha$ -synuclein (3), and this may be related to its toxicity. Alternatively, inhibitory effects of the S129D mutant on intracellular proteasome activity, as shown in this study, may be involved in cell death.

Importantly, we have shown that intracellular  $\alpha$ -synuclein may be toxic to neuronal cells because the protein causes proteasome dysfunction. It was previously reported that duplication and triplication of  $\alpha$ -synuclein gene causes familial PD (7–11), indicating that overproduction of  $\alpha$ -synuclein protein could lead to the onset of PD. It is reasonable to speculate that proteasome activity may also be affected in neurons of these patients in a manner similar to that shown in this study. It may be important to prevent  $\alpha$ -synuclein-induced inhibition or decrease of proteasome activity. In this study, we screened for compounds protecting cells from the  $\alpha$ -synuclein-mediated proteasome dysfunction and found that purpurogallin, myricetin, exifone, and gossypetin (polyphenols from plants) prevented proteasome impairment by  $\alpha$ -synuclein. These compounds are thought to inhibit *in vitro*  $\alpha$ -synuclein filament formation by stabilizing soluble prefibrillar intermediates (35). We speculate that these small molecules may interact with oligomeric forms of  $\alpha$ -synuclein in cultured cells, although we can not exclude the possibility that these compounds directly regulate the activity of the proteasome. The precise mechanisms by which these polyphenols prevent  $\alpha$ -synuclein-mediated proteasome dysfunction remain unknown. However, these compounds may be novel drug candidates for neurodegenerative diseases. Our cellular model system using coexpression of GFP-CL1 and  $\alpha$ -synuclein should be a sensitive and useful tool for screening of compounds and drugs for the prevention and treatment of these disease.

## ACKNOWLEDGMENT

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## SUPPORTING INFORMATION AVAILABLE

Fractionation of cells expressing both of GFP-CL1 and  $\alpha$ -synuclein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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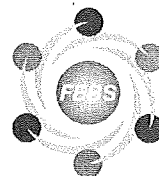
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## Inhibition of $\alpha$ -synuclein fibril assembly by small molecules: Analysis using epitope-specific antibodies

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### ABSTRACT

**The conversion of soluble peptides and proteins into amyloid fibrils and/or intermediate oligomers is believed to be the central event in the pathogenesis of most human neurodegenerative diseases. Existing treatments are at best symptomatic. Accordingly, small molecule inhibitors of amyloid fibril formation and their mechanisms are of great interest. Here we report that the conformational changes undergone by  $\alpha$ -synuclein as it assembles into amyloid fibrils can be detected by epitope-specific antibodies. We show that the conformations of polyphenol-bound  $\alpha$ -synuclein monomers and dimers differ from those of unbound monomers and resemble amyloid fibrils. This strongly suggests that small molecule inhibitors bind and stabilize intermediates of amyloid fibril formation, consistent with the view that inhibitor-bound molecular species are on-pathway intermediates.**

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### 1. Introduction

The conversion of a small number of soluble peptides and proteins into amyloid fibrils and/or intermediate oligomers is believed to be the central event in the pathogenesis of most neurodegenerative diseases. Three proteins,  $\beta$ -amyloid, tau and  $\alpha$ -synuclein, make up the abnormal deposits in the vast majority of disease cases [1,2]. Many current therapeutic strategies are aimed at inhibiting filament formation and at promoting filament clearance. In recent years, a number of compounds has been identified that prevent amyloid fibril formation in vitro [3–10]. In the absence of effective therapies for neurodegenerative diseases, it is important to understand the mechanisms of action of these compounds.

We previously reported that non-toxic, SDS-stable dimers and oligomers of tau and  $\alpha$ -synuclein formed in the presence of inhibitory compounds and that their formation closely correlated with the inhibition of fibril formation [8,9]. This suggests that small

molecule inhibitors stabilize non-toxic, on-pathway intermediates. Based on these observations, we presented a model for the inhibition of tau,  $\alpha$ -synuclein and A $\beta$  aggregation by small molecules. Ehrnhoefer et al. recently presented a different model for the inhibition of fibril formation of  $\alpha$ -synuclein and A $\beta$  by the small molecule inhibitor (–)-epigallocatechin-3-gallate (EGCG) [11]. They reported that EGCG inhibited the assembly of  $\alpha$ -synuclein and A $\beta$  by binding to natively unfolded protein monomers, preventing their conversion into toxic on-pathway aggregation intermediates. Instead,  $\alpha$ -synuclein and A $\beta$  formed unstructured, non-toxic oligomers that were said to be off-pathway.

In this study, we used 10 epitope-specific antibodies of  $\alpha$ -synuclein spanning the whole of  $\alpha$ -synuclein and investigated their reactivities with monomeric  $\alpha$ -synuclein and with  $\alpha$ -synuclein fibrils. Some antibodies detected conformational changes that distinguished monomers from fibrils. These antibodies were then used to detect conformational changes in polyphenol-stabilized monomers and dimers of  $\alpha$ -synuclein. Importantly, SDS-stable, polyphenol-stabilized monomers and dimers showed an intermediate reactivity between that of monomers and fibrils. These findings indicate that inhibitory compounds bind and stabilize on-pathway intermediates of amyloid fibril formation.

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