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Casein Kinase II-mediated Phosphorylation Regulates α-Synuclein/ Synphilin-1 Interaction and Inclusion Body Formation*

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α-Synuclein is a phosphoprotein that accumulates as a major component of Lewy bodies in the brains of patients with Parkinson disease. Synphilin-1, which is also present in Lewy bodies, binds with α-synuclein and forms cytoplasmic inclusions in transfected cells. Yet the molecular determinants of this protein-protein interaction are unknown. Here we report that casein kinase II (CKII) phosphorylates synphilin-1 and that the β subunit of this enzyme complex binds to synphilin-1. Additionally, both CKII α and β subunits are present within cytoplasmic inclusions in cells that overexpress synphilin-1. Notably, the interaction between synphilin-1 and α-synuclein is markedly dependent on phosphorylation. Inhibition of CKII activity by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole blocks the binding between these two proteins and significantly reduces the percentage of cells that contain eosinophilic cytoplasmic inclusions. Mutation of the major CKII phosphorylation site in α-synuclein (S129A) has no significant impact on the binding between α-synuclein and synphilin-1 or on the formation of synphilin-1/α-synuclein-positive inclusions. These data suggest that the CKII-mediated phosphorylation of synphilin-1 rather than that of α-synuclein is critical in modulating their tendency to aggregate into inclusions. These observations collectively indicate that a ubiquitous post-translational modification such as phosphorylation can regulate inclusion body formation in the context of α-synuclein and synphilin-1 interaction.

Parkinson disease (PD)[1] is a common neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. Accumulating evidence suggests that aberrations in protein processing or folding leading to the aggregation of pathogenic proteins or their partners are a common theme in many degenerative disorders affecting the brain, including PD (1–3). Lewy bodies, classically considered as pathological hallmark features of PD, are proteinaceous cytoplasmic inclusions that consist of many components, among which α-synuclein is a major constituent (4, 5). The first indication for the pathogenic role of α-synuclein in PD came from the linkage of mutations in its gene with autosomal dominant forms of PD (6, 7). Another component of Lewy bodies is synphilin-1, which was discovered by screening for proteins that interact with α-synuclein (8). Co-expression of these two proteins results in the formation of cytoplasmic inclusions in a small percentage of cultured cells (8, 9). But the molecular determinants that regulate this interaction and perhaps consequent inclusion body formation are unknown. Such factors would elucidate not only the genesis of these aggregates but could also provide clues about potential therapeutic targets if the inclusions or their precursors are pathogenic.

Phosphorylation is a common post-translational modification that regulates the function and other properties of many proteins. α-Synuclein itself is phosphorylated both *in vitro* and *in vivo* by casein kinase (CK) I and II (10). Additionally, α-synuclein is extensively phosphorylated at serine 129 in synucleinopathy lesions and in overexpressing transgenic mice and flies (11–13). Yet, the physiological significance of this phosphorylation and its link to the pathogenesis of PD are unclear. Synphilin-1 also has consensus sequences for putative CKII recognition sites, but whether it is indeed phosphorylated has not been addressed. In this investigation we studied the phosphorylation of synphilin-1 by CKII and elucidated the critical role of this post-translational modification in the interaction between synphilin-1 and α-synuclein and in inclusion body formation.

EXPERIMENTAL PROCEDURES

Construction of Synphilin-1 and CKII Expression Vectors—pFLAG-synph expressing full-length synphilin-1 with an N-terminal FLAG tag was generated as described previously (14). α-Synuclein cDNA was amplified by PCR from a human brain cDNA library (Stratagene) and inserted into pcDNA3.1 expression vector (Invitrogen) to generate pSyn-WT. To generate α-synuclein cDNA with a serine 129-alanine substitution, pSyn-WT was used as a template to introduce the T385G point mutation using the QuickChange site-directed mutagenesis kit (Stratagene). To generate an expression vector for myc-tagged casein kinase II β subunit, the entire open reading frame was amplified by PCR from a human brain cDNA library (Invitrogen) using primers

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[1] The abbreviations used are: PD, Parkinson disease; synph-293, human embryonic kidney 293T cells transfected with pFLAG-synphilin-1; CK, casein kinase; PBS, phosphate-buffered saline; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

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5′-cccgggCGTGAAGATGAGCA-3′ and 5′-agatctGGGAGGGAATCAG-C-3′ (SmaI and BglII sites in small characters were incorporated to facilitate cloning). The product was subcloned in pCMV-Tag3 (Stratagene) for generating pMyc-CKII β, which expresses CKII β with an N-terminal myc tag. The integrity of all constructs was verified by DNA sequencing.

Cell Culture, Transfection, and Generation of Stable Cell Lines—293T and PC-12 cells were obtained from the American Type Culture Collection. NS20Y cells were a kind gift from Dr. Marshall Nirenberg (NHLBI, National Institutes of Health, Bethesda, MD). All cell lines except PC-12 were cultured in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum (BioWhittaker) and grown at 37 °C in a humidified atmosphere containing 10% CO_2. PC-12 cells were grown in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% fetal bovine serum. The 293T cells were transfected with pFLAG-synphilin, selected, cloned, and maintained in medium containing 1 mg/ml G418 (Invitrogen) to generate synph-293 cells. All transfections used the calcium phosphate transfection kit (Invitrogen) according to the supplier's instructions.

In Vitro Phosphorylation—Synph-293 cells constitutively expressing FLAG-tagged synphilin-1 were harvested by scraping and were centrifuged at 3000 × *g* for 1 min at 4 °C. The cell pellet was washed with Dulbecco's phosphate-buffered saline (PBS) twice and resuspended in lysis buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 10% glycerol, 150 mM NaCl, 0.5% Nonidet P-40, 2 mM sodium vanadate, 10 mM NaF, and 10 mM sodium pyrophosphate with a protease inhibitor mixture, Set III (Calbiochem). FLAG-tagged synphilin-1 was immunoprecipitated by anti-FLAG M2 agarose-affinity gel (Sigma) and incubated in 40 μl of kinase buffer (20 mM Tris-HCl (pH 7.5), 50 mM KCl, and 10 mM $MgCl_2$). The reaction was started by the addition of 10 μl of a mixture of 100 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 250 units of CKII (New England Biolabs), and the mixture was incubated at 30 °C for 30 min. The reaction was stopped by adding SDS gel-loading buffer (Invitrogen) and boiling at 95 °C for 5 min. Proteins were separated by SDS-PAGE, and synphilin-1 phosphorylation was analyzed by autoradiography.

In Vivo Phosphorylation—Synph-293 cells stably expressing FLAG-tagged synphilin-1 and NS20Y cells transiently transfected with pFLAG-synphilin were grown to subconfluence and incubated in phosphate-free medium (Invitrogen) for 1 h. This medium was replaced with fresh phosphate-free medium containing 20–35 MBq of [^{32}P]orthophosphate, and cells were incubated at 37 °C for 4 h. After removing the radioactive medium, the cells were washed three times with ice-cold PBS, immediately scraped off the plate, and lysed on ice with a lysis buffer. Lysates were centrifuged at 12,000 × *g* for 10 min, and supernatants were immunoprecipitated with anti-FLAG M2 antibody for 6 h. Proteins were separated by SDS-PAGE, and synphilin-1 phosphorylation was analyzed by autoradiography.

Immunoprecipitation and Western Blots—For co-immunoprecipitation, synph-293 cells were transfected with pSyn-WT or pSyn-S129A. After 48 h, FLAG-tagged synphilin-1 was immunoprecipitated by anti-FLAG M2 agarose-affinity gel (Sigma) as described above. The precipitates or total cell lysates were subjected to Western blot analysis with anti-α-synuclein polyclonal antibody (1:2000) (15) or anti-FLAG M2 antibody (1:5000) (Sigma).

Immunocytochemistry and Quantification of Inclusions—The antibodies used for immunocytochemistry were α-synuclein monoclonal (1:400) (Zymed Laboratories Inc.), FLAG Cy3-conjugated monoclonal (1:1000) (Sigma), casein kinase II α subunit (1:80) (Calbiochem), casein kinase II β subunit (1:100, Calbiochem), fluorescein isothiocyanate-secondary antibody (1:100) (Vector Laboratories), and Texas Red-secondary antibody (1:100) (Vector Laboratories). After being washed with PBS, the cells were fixed in 4% formaldehyde for 10 min at room temperature, permeabilized with 0.3% Triton X-100 for 15 min, and blocked with 3% bovine serum albumin for 30 min. Cells were then incubated at 4 °C for 24 h with the appropriate primary antibody and diluted in PBS containing 1% bovine serum albumin. After the cells were washed three times with PBS, the secondary antibody, diluted in PBS and containing 1% bovine serum albumin, was added for 2 h at room temperature. Samples were visualized with a fluorescence confocal microscope (LSM 510, Zeiss).

For quantification of synphilin-1-positive inclusions, transfected cells were immunostained with anti-FLAG Cy3-conjugated antibody diluted in 1% bovine serum albumin in PBS for 3 h at 4 °C and counted under a fluorescence microscope (Optiphot-2, Nikon). Two hundred transfected cells in randomly selected fields were counted for each condition in triplicate experiments. Inclusion-bearing cells were also quantified using hematoxylin-eosin staining (Fisher Scientific) for identifying eosinophilic inclusions. Cells were counted in three to five randomly selected fields of the chamber (Collagen I Cellware 4 Well CultureSlide, BD Biosciences) by blind investigation. A total of 12 wells and 1200 cells per condition were counted.

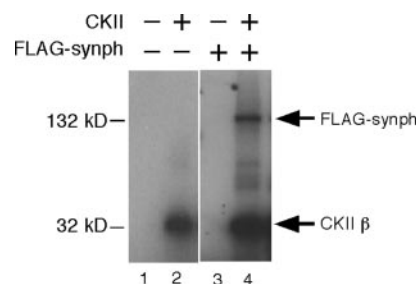

FIG. 1. ***In vitro* phosphorylation of synphilin-1 by CKII.** FLAG-tagged synphilin-1 was immunoprecipitated from synph-293 cells with anti-FLAG antibody and incubated with [γ-^{32}P]ATP in a CKII reaction buffer. Labeled proteins were separated by SDS-PAGE, and phosphorylated synphilin-1 was visualized by autoradiography. The known autophosphorylation of CKII β is seen in *lanes 2* and *4*.

RESULTS

Synphilin-1 Is Phosphorylated by CKII Both in Vitro and in Vivo—The amino acid sequence of synphilin-1 includes 19 putative phosphorylation sites for casein kinase II (S/T)*XX*(D/E) (16). Thus, the possibility that synphilin-1 is phosphorylated by CKII was tested. First, *in vitro* kination assays were performed using FLAG-tagged synphilin-1 immunoprecipitated with anti-FLAG M2 antibody from stably transfected 293T (synph-293) cells as a substrate in the presence of human CKII and [γ-^{32}P]ATP. Autoradiography of electrophoresed proteins revealed that synphilin-1 is strongly phosphorylated, whereas no phosphorylated synphilin-1 was observed in the absence of CKII (Fig. 1, *lanes 3* and *4*).

Next, we investigated whether synphilin-1 is phosphorylated in synph-293 cells and in transiently transfected NS20Y cells. These cells were incubated with [^{32}P]orthophosphate in the presence or absence of the CKII inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or the phosphatase inhibitor okadaic acid. FLAG-tagged synphilin-1 was immunoprecipitated with anti-FLAG M2 antibody, and phosphorylated synphilin-1 was visualized by autoradiography. In the presence of DRB, synphilin-1 phosphorylation was strongly inhibited in synph-293 cells (Fig. 2*A*) and was diminished dose-dependently in NS20Y cells (Fig. 2*B*). Thus, synphilin-1 is phosphorylated by CKII in cells of neuronal and non-neuronal origin. Treatment of synph-293 cells with okadaic acid resulted in a substantial increase of phosphorylated synphilin-1 to a level much higher than its base line (Fig. 2*A*). These findings suggest that synphilin-1 is highly phosphorylated and rapidly dephosphorylated *in vivo*.

Regulatory β Subunit of CKII Interacts with Synphilin-1—CKII-mediated phosphorylation requires the association of its β subunit with substrate proteins (17). To confirm that such an interaction also exists with synphilin-1, we examined the binding between synphilin-1 and the CKII β subunit. We transfected synph-293 cells with myc-tagged CKII β subunits and subjected lysates to immunoprecipitation with anti-myc antibody followed by anti-FLAG immunoblotting (Fig. 3*A*). The CKII β subunit co-immunoprecipitated with synphilin-1 in a specific manner, suggesting that the two molecules did bind. To exclude the possibility that this finding was simply the result of CKII β overexpression, we checked for binding between endogenous CKII β and synphilin-1 in PC-12 cells transiently transfected only with pFLAG-synphilin. FLAG-tagged synphilin-1 was immunoprecipitated by anti-FLAG antibody, and the electrophoresed proteins were subjected to Western blotting with antibodies to CKII α and β. As expected, a specific interaction

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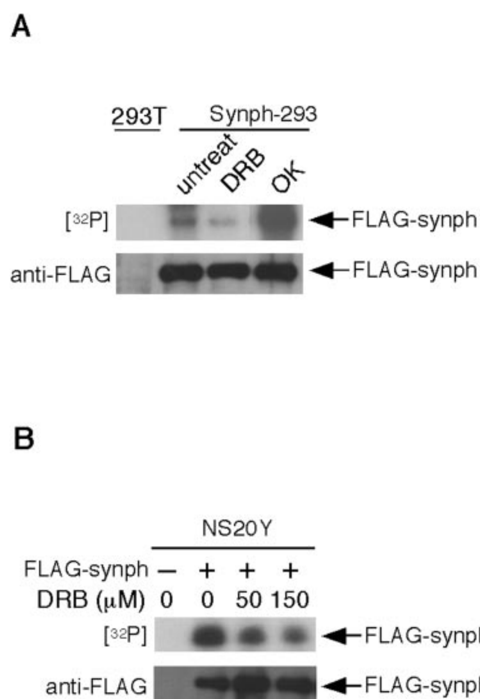

FIG. 2. ***In vivo* phosphorylation of synphilin-1 by CKII.** *A*, synphilin-1 is phosphorylated by CKII in 293T cells. Synph-293 cells stably overexpressing FLAG-tagged synphilin-1 were incubated with [^{32}P]orthophosphate in the presence of 50 μM DRB or 0.2 μM okadaic acid (*OK*). Lysates were subjected to immunoprecipitation with anti-FLAG M2 antibody, and phosphorylated FLAG-synphilin-1 was visualized by autoradiography. Native 293T cells are shown as control. The *lower panel* shows equal expression of total FLAG-synphilin-1 in all synph-293 cells by Western blot using anti-FLAG antibody. *B*, synphilin-1 is phosphorylated by CKII in NS20Y cells transiently transfected with FLAG-tagged synphilin-1. ^{32}P incorporation into synphilin-1 decreased dose dependently with different amounts of DRB. The *lower panel* shows total expression of FLAG-synphilin-1 by Western blot using anti-FLAG antibody.

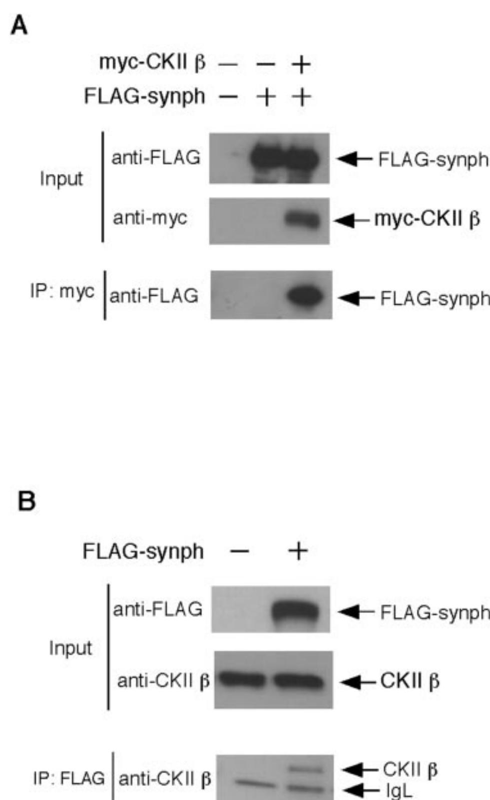

FIG. 3. **The β subunit of CKII interacts with synphilin-1.** *A*, synph-293 cells were transiently transfected with pMyc-CKII β. Lysates were subjected to immunoprecipitation with anti-myc 9E10 antibody followed by anti-FLAG immunoblotting (*lower panel*). The *upper two panels* show total expression of FLAG-synphilin-1 and myc-CKII β in the lysate. *B*, endogenous CKII β interacts with synphilin-1 in PC-12 cells. These cells were transiently transfected with FLAG-tagged synphilin-1. Lysates were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-CKII β E-19 antibody (*lower panel*). The *two upper panels* show FLAG-synphilin-1 and CKII β expression in total lysate. *IgL*, IgG light chains.

was observed between synphilin-1 and endogenous CKII β (Fig. 3*B*), whereas no such interaction was detected with the catalytic subunit CKII α (data not shown). We conclude that CKII β first recognizes synphilin-1, and then CKII α phosphorylates this substrate.

CKII Co-localizes with α-Synuclein in Cytoplasmic Inclusions—Based on the ability of CKII to phosphorylate α-synuclein (10) and synphilin-1 (Figs. 1 and 2), we next verified the microscopic localization of CKII subunits within inclusions formed by these substrates. Immunocytochemical staining of synph-293 cells revealed CKII β immunoreactivity (visualized with fluorescein isothiocyanate-conjugated secondary antibody), which was co-localized with anti-α-synuclein (visualized by Texas Red-conjugated secondary antibody) within cytoplasmic inclusions (Fig. 4*B*). Similarly, the CKII α subunit co-localized with α-synuclein in these inclusions (Fig. 4*A*).

Effect of CKII-mediated Phosphorylation on the Binding between Synphilin-1 and α-Synuclein—Because synphilin-1 and α-synuclein are interacting partners (8) and because both are phosphorylated by CKII (see data above and Ref. 10), we tested whether modulating the activity of this enzyme could influence the binding between these two proteins. Synph-293 cells were transiently transfected with α-synuclein (18) followed by treatment with different amounts of DRB. Lysates were subjected to immunoprecipitation with anti-FLAG antibody and were immunoblotted with anti-α-synuclein antibody. Inhibition of CKII by DRB resulted in a marked reduction of the binding between α-synuclein and synphilin-1 (Fig. 5), indicating that CKII-mediated phosphorylation is a potent regulator of this interaction. Inhibition of phosphorylation did not have a significant impact on total intracellular levels of synphilin-1 or α-synuclein (Fig. 5, *upper two panels*).

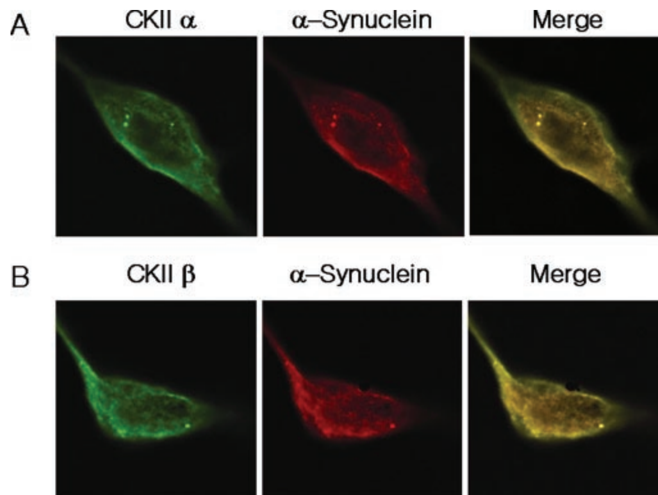

FIG. 4. **CKII subunits co-localize with α-synuclein in cytoplasmic inclusions.** *A*, synph-293 cells were stained for CKII α (fluorescein isothiocyanate, *green*) and α-synuclein (Texas Red, *red*). *B*, synph-293 cells were stained for CKII β (fluorescein isothiocyanate, *green*) and α-synuclein (Texas Red, *red*).

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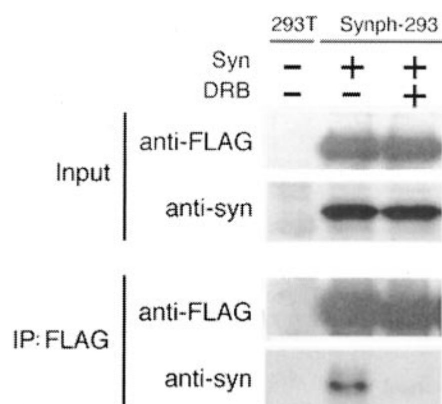

FIG. 5. **CKII-mediated phosphorylation regulates the interaction between synphilin-1 and α-synuclein.** Synph-293 cells were transiently transfected with α-synuclein followed by treatment with 50 μM DRB for 24 h. Lysates were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-FLAG antibody or with anti-α-synuclein polyclonal antibody (*two lower panels*). The *two upper panels* show the expression of the two transgenes in total lysate. Native 293T cells were used as a control.

Inhibition of CKII Activity Reduces Eosinophilic Cytoplasmic Inclusions—Co-expression of α-synuclein and synphilin-1 results in the formation of eosinophilic cytoplasmic inclusions in cellular models (8). To test the effect of phosphorylation by CKII on the formation of such cytoplasmic inclusions, synph-293 cells were transiently transfected with α-synuclein followed by treatment with 50 μM DRB and stained with hematoxylin-eosin (Fig. 6). Analysis of synph-293 cells overexpressing only synphilin-1 revealed that 4.3% of total cells had eosinophilic cytoplasmic inclusions (Fig. 6, *A* and *C*). As expected, co-expression of both synphilin-1 and α-synuclein increased the number of cells with inclusions to 7.6%. Notably, treatment of cells co-expressing both proteins with DRB significantly reduced the percentage of inclusion-bearing cells to 4.6% (Fig. 6*C*). The latter finding is consistent with the inhibition of the synphilin-1/α-synuclein interaction by DRB on Western blot analysis (Fig. 5). The number of inclusions in synph-293 cells in the absence of ectopic α-synuclein overexpression did not change significantly after DRB treatment. These observations led us to conclude that the inhibition of CKII activity suppresses the co-aggregation of synphilin-1 and α-synuclein into inclusion bodies.

Phosphorylation of α-Synuclein by CKII Is Not Important for Regulating Its Binding with Synphilin-1—The above data demonstrated that CKII-mediated phosphorylation regulates the formation of α-synuclein/synphilin-1-positive inclusions (Figs. 5 and 6). However, whether the phosphorylation of synphilin-1 or α-synuclein, or both, is important for inclusion body formation is not clear. Serine 129 is a major CKII phosphorylation site within the C-terminal domain of α-synuclein (10), whereas the functional phosphorylated residue(s) in synphilin-1 is not determined. To investigate the importance of α-synuclein phosphorylation in this protein-protein interaction, we generated an α-synuclein mutant cDNA that harbors a serine-to-alanine substitution at position 129 (S129A), rendering this major CKII phosphorylation site nonfunctional. When synph-293 cells were transiently transfected with wild-type or S129A mutant α-synuclein, immunoprecipitation followed by Western blotting revealed that the interaction of the mutant α-synuclein isoform with synphilin-1 is not compromised (Fig. 7). This finding indicates that α-synuclein phosphorylation by CKII has no significant effect on its binding with synphilin-1. A similar result was obtained in yeast with two hybrid experiments (data not shown). Based on these observations we conclude that it is the

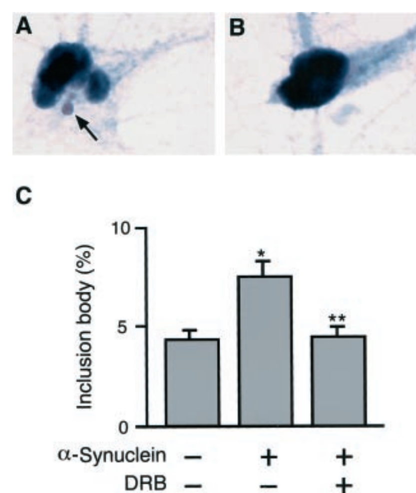

FIG. 6. **Cytoplasmic inclusions formed by synphilin-1 and α-synuclein are subject to regulation by phosphorylation.** *A* and *B*, synph-293 cells were transiently transfected with α-synuclein for 24 h and stained with hematoxylin-eosin. A cell with a single eosinophilic cytoplasmic inclusion is shown in *A* (*arrow*). A control cell without inclusion is shown in *B*. *C*, quantification of eosinophilic inclusions in synph-293 cells transiently transfected with α-synuclein or its empty vector, followed by treatment with 50 μM DRB or vehicle for 24 h is shown. *, analysis of variance $p < 0.002$ compared with no α-synuclein overexpression; **, $p < 0.003$ compared with α-synuclein transfected cells but no DRB treatment. Data shown are means ± S.E. for four independent experiments.

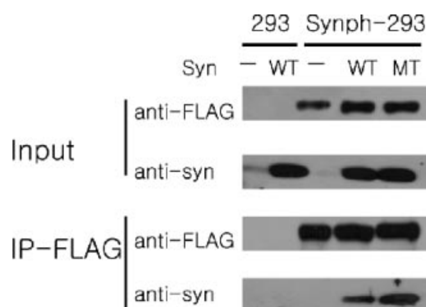

FIG. 7. **Phosphorylation of synphilin-1 regulates the interaction between synphilin-1 and α-synuclein.** Synph-293 cells were transiently transfected with wild-type or S129A mutant α-synuclein for 48 h. Lysates were subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-FLAG antibody (*two upper panels*) or with anti-α-synuclein polyclonal antibody (*two lower panels*). Native 293T cells were used as a control.

CKII-mediated phosphorylation of synphilin-1 rather than the phosphorylation of α-synuclein that is critical for the interaction between these two protein partners.

Phosphorylation of α-Synuclein at Serine 129 Does Not Influence Its Aggregation with Synphilin-1 in Cytoplasmic Inclusions—To further investigate the effect of α-synuclein phosphorylation on the formation of cytoplasmic inclusions with synphilin-1, we quantified inclusion-bearing synph-293 cells transiently transfected with wild-type or S129A mutant α-synuclein using immunocytochemistry (Fig. 8). In these experiments, approximately 10% of synph-293 cells transfected with wild-type α-synuclein contained cytoplasmic inclusions based on immunoreactivity for FLAG-tagged synphilin-1. Synphilin-293 cells transfected with S129A mutant α-synuclein showed a comparable percentage of inclusion formation, confirming that the phosphorylation of α-synuclein at serine 129 is not critical for its tendency to form inclusions with synphilin-1.

DISCUSSION

We have found that the α-synuclein partner, synphilin-1, interacts with the regulatory subunit of the serine-threonine

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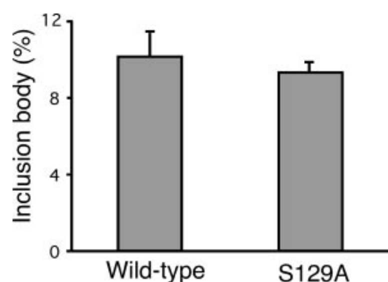

FIG. 8. **Phosphorylation of α-synuclein at serine 129 does not influence the formation of synphilin-1/α-synuclein-immunoreactive inclusions.** Synph-293 cells were transiently transfected with wild-type or S129A (*S129A*) mutant α-synuclein and cultured for 24 h. Cytoplasmic inclusions were detected by immunostaining for FLAG-synphilin-1. Data shown are means ± S.E. for three independent experiments.

kinase CKII and is phosphorylated by this kinase complex both *in vitro* and *in vivo*. Furthermore, we have demonstrated that the interaction between α-synuclein and synphilin-1 is tightly regulated by the phosphorylation of synphilin-1. Inhibition of this post-translational modification potently dissociates protein-protein complexes and diminishes their ability to aggregate into cytoplasmic inclusions.

Phosphorylation plays an important role in the pathogenesis of certain neurodegenerative diseases. Alzheimer disease is the best recognized example of a brain disorder in which phosphorylation of a specific protein plays a pathogenetic role, and there are other conditions associated with neurofibrillary tangles (19). In Alzheimer disease, hyperphosphorylation prevents the microtubule-associated protein tau from assembling normally with microtubules and the orderly polymerization of microtubules. Thus, hyperphosphorylated tau proteins become the main components of the aggregated filaments found in neurofibrillary tangles (20, 21). Recently, extensively phosphorylated α-synuclein at serine 129 was also described in synucleinopathy lesions with Lewy bodies in the brains of patients with dementia (11). Similar observations have been made in α-synuclein transgenic *Drosophila* (13) and in A30P mutant transgenic mice (12). Our observations indicate that the phosphorylation of synphilin-1 is more critical in the interaction with α-synuclein in forming inclusions than for the phosphorylation of α-synuclein. It is conceivable that the localization of synphilin-1 mainly in the core of Lewy bodies (22), surrounded by the halo where α-synuclein is mostly present (23), may protect this large protein from phosphatases, although the status of its phosphorylation *in vivo* remains to be studied.

CKII is a ubiquitous and pleiotropic serine-threonine protein kinase, which is highly conserved in evolution, indicating its vital biological roles (24). Its activity and expression are relatively high in the brain (25, 26) where it is distributed in most regions and localized primarily in neurons (27, 28). CKII has myriad substrates and consequently controls many neuronal functions including development, neuritogenesis, synaptic transmission, plasticity, information storage, and survival (29). Additionally, phosphorylation by CKII could be implemented in the genesis of certain neuronal cytoplasmic aggregates. In Alzheimer disease, for example, increased CKII immunolabeling has been demonstrated in neurofibrillary tangle-bearing neurons (30, 31). The association of CKII with tangles and other inclusions has also been reported in other neurodegenerative disorders including the Guamanian Parkinson dementia complex, progressive supranuclear palsy, and Pick disease (32).

The co-aggregation of α-synuclein and synphilin-1 leads to the formation of cytosolic inclusions both in non-neuronal 293T cells and in neuronally derived SH-SY5Y cells (data not shown). In 293T cells stably transformed to express synphilin-1, 4.3% manifest eosinophilic cytoplasmic inclusions. Whether these represent an aggregation of synphilin-1 alone or seeding by low levels of endogenous α-synuclein (33) requires further investigation. The percentage of inclusion-positive cells in our experiments is higher than that obtained following transient co-transfection of α-synuclein and synphilin-1 (less than 1%) in previous studies (8, 9). This quantitative discrepancy can be attributed to the fact that our cells are stably engineered with synphilin-1 and are cloned. Transient transfection of synph-293 cells with α-synuclein leads to a further increase of inclusion-positive cells to approximately 8–10%, which is demonstrated by both hematoxylin-eosin staining and by fluorescent immunocytochemistry. These quantitative findings were highly reproducible in our system. Thus, the intracellular levels of these proteins are crucial for their aggregation into inclusions. This latter notion is consistent with the fact that transgenic mice and flies with increased α-synuclein expression develop neuronal inclusions in dopaminergic neurons (34, 35).

Neuronal inclusions in neurodegenerative diseases, such as Lewy bodies in PD, had for decades been no more than pathological diagnostic aids; but recent advances in the genetics and molecular biology of these disorders have made it possible to study their genesis and more importantly their role in neuronal death. The question of whether intracellular inclusions protect against or contribute to cytotoxicity has been debated (36). Our recent quantitative analysis of cells with and without inclusions containing α-synuclein/synphilin-1 demonstrated that such microscopically visible inclusions are present mostly in surviving cells and less so in apoptotic cells (37). Furthermore, cells containing inclusions have normal morphology and intact nuclei with no evidence of caspase activation (data not shown), suggesting that the inclusions *per se* are not cytotoxic. These findings are consistent with experimental evidence from cellular and transgenic animal models of polyglutamine expansion disorders, indicating a lack of correlation between inclusions and neuronal death (38–40). *In vitro* structural studies of α-synuclein have revealed various conformations ranging from smaller oligomers to more complex fibrils and have raised the possibility that cytotoxicity is not the result of the fibrils that form Lewy bodies but rather because of their oligomeric precursors that remain in the cytoplasm (41).

The above observations collectively suggest that the interaction between α-synuclein and synphilin-1 as well as subsequent inclusion body formation are regulated by the phosphorylation of synphilin-1. If the submicroscopic interaction between these two proteins is of critical importance in the pathogenesis of dopaminergic neuronal death in Parkinson disease, targeted pharmacological disruption of binding through manipulating phosphorylation could have therapeutic value.

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