Glycogen Synthase Kinase 3β Modulates Synphilin-1 Ubiquitylation and Cellular Inclusion Formation by SIAH

IMPLICATIONS FOR PROTEASOMAL FUNCTION AND LEWY BODY FORMATION*

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α-Synuclein is known to play a major role in the pathogenesis of Parkinson disease. We previously identified synphilin-1 as an α-synuclein-interacting protein and more recently found that synphilin-1 also interacts with the E3 ubiquitin ligases SIAH-1 and SIAH-2. SIAH proteins ubiquitylate synphilin-1 and promote its degradation through the ubiquitin proteasome system. Inability of the proteasome to degrade synphilin-1 promotes the formation of ubiquitylated inclusion bodies. We now show that synphilin-1 is phosphorylated by GSK3β within amino acids 550–659 and that this phosphorylation is significantly decreased by pharmacological inhibition of GSK3β and suppression of GSK3β expression by small interfering RNA duplex. Mutation analysis showed that Ser556 is a major GSK3β phosphorylation site in synphilin-1. GSK3β co-immunoprecipitated with synphilin-1, and protein 14-3-3, an activator of GSK3β activity, increased synphilin-1 phosphorylation. GSK3β decreased the in vitro and in vivo ubiquitylation of synphilin-1 as well as its degradation promoted by SIAH. Pharmacological inhibition and small interfering RNA suppression of GSK3β greatly increased ubiquitylation and inclusion body formation by SIAH. Additionally, synphilin-1 S556A mutant, which is less phosphorylated by GSK3β, formed more inclusion bodies than wild type synphilin-1. Inhibition of GSK3β in primary neuronal cultures decreased the levels of endogenous synphilin-1, indicating that synphilin-1 is a physiologic substrate of GSK3β. Using GFPu as a reporter to measure proteasome function in vivo, we found that synphilin-1 S556A is more efficient in inhibiting the proteasome than wild type synphilin-1, raising the possibility that the degree of synphilin-1 phosphorylation may regulate the proteasome function. Activation of GSK3β during endoplasmic reticulum stress and the specific phosphorylation of synphilin-1 by GSK3β place synphilin-1 as a possible mediator of endoplasmic reticulum stress and proteasomal dysfunction observed in Parkinson disease.

Parkinson disease (PD) is characterized by loss of dopaminergic neurons in the substantia nigra and the presence of cytoplasmic inclusions called Lewy bodies in surviving neurons (1). Hereditary PD can be caused by mutations in the α-synuclein gene (2–4) and in components of the ubiquitin-proteasome system, such as the E3 ubiquitin ligase parkin and UCH-L1 (5, 6).

α-Synuclein is a major component of Lewy bodies in sporadic PD (7). Overexpression of α-synuclein inhibits the proteasomal activity (8–10) and causes cell death in a variety of cell and animal models (11). In agreement, proteasomal activity is decreased in substantia nigra of PD patients (12).

We have shown that synphilin-1 is a presynaptic protein that interacts with α-synuclein in vivo (13, 14). Synphilin-1 leads to the formation of inclusion bodies when co-transfected with the non-α component portion of α-synuclein in cultured cells and is an intrinsic component of Lewy bodies in PD, suggesting that it may play a role in Lewy body formation (13, 15). Synphilin-1 seems to have a dual role in cell survival. Synphilin-1 is toxic to cells and inhibits proteasomal activity, raising the possibility that synphilin-1 might contribute to the death of dopaminergic neurons in PD (16–18). On the other hand, cells containing synphilin-1 inclusions are more resistant to death, indicating that inclusions might be neuroprotective (19–21). Additional evidence that synphilin-1 may be involved in PD comes from identification of a missense mutation in its gene in two patients that share a rare haplotype (20).

It has been shown that Lewy bodies are ubiquitylated, and understanding the ubiquitylation mechanism of Lewy body proteins may be relevant for clarifying ubiquitin’s role in Lewy body formation. We have recently reported that the E3 ubiquitin ligase SIAH-1 and SIAH-2 ubiquitylate and target synphilin-1 for degradation by the proteasome system (19). The inability of the proteasome to degrade the synphilin-1-SIAH complex leads to a robust formation of ubiquitylated cytosolic inclusions containing synphilin-1, SIAH, and α-synuclein (19). Ubiquitylation is required for inclusion body formation, since a catalytically inactive mutant of SIAH-1 that binds to synphilin-1 fails to promote inclusions (19). Additionally, both SIAH and synphilin-1 are present in Lewy bodies of PD patients, implying a role in inclusion formation.

In an attempt to better understand the role of synphilin-1 in PD and Lewy body formation, we now sought to investigate mechanisms that regulate synphilin-1 ubiquitylation and inclusion formation. We present data indicating that synphilin-1 is phosphorylated in vivo by GSK3β, which regulates ubiquitin-dependent degradation of synphilin-1 and inclusion body formation mediated by SIAH. Selective inhibition of GSK3β or mutation of a GSK3β phosphorylation site greatly increased synphilin-1 aggregation into cytosolic inclusions, suggesting a role of phosphorylation in modulating synphilin-1 aggregation. GSK3β inhibitor also enhanced the degradation of endogenous synphilin-1 in neurons, indicating that synphilin-1 is a physiologic substrate of GSK3β. We also present data indicating that inhibition of proteasome function by synphilin-1 is modulated by its phosphorylation status. Our results shed light on the mechanism regulating synphilin-1 ubiquitylation and...
aggregation, with implications for inclusion body formation and possibly cell death in PD.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—Ubiquitin aldehyde and purified ubiquitin-activating enzyme were purchased from Boston Biochem. [35S]Methionine/cysteine, [32P]orthophosphate, and [32P]ATP were purchased from Amer sham Biosciences. 5,6-Dibromo-1-β-D-ribofuranosylbenzimidazole (DRB), SB415286, kenpaullone, and other protein kinase inhibitors were purchased from Sigma.

**Cell Culture and Transfections**—HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a 5% CO2 atmosphere. Cells were transiently transfected with N-termi nally tagged pRK5 and pFLAG-CMV-2 plasmids utilizing Lipofectamine 2000 (Invitrogen) and processed after 36 h.

For experiments using siRNA, HEK 293 cells were transfected with 50 nM siRNAs using Lipofectamine 2000. After 48 h, cells were transfected with HA-synphilin-1 constructs and additional 50 nM siRNAs and processed after 36 h. Silencer-validated siRNA to GSK3β (sense siRNA strand, 5’-GGCAGCAGGAUAAAGAATT-3’), antisense siRNA strand, 5’-AUUUCUUAUACUCUGCCCTG-3’) and negative control siRNA 1 (sense siRNA strand, 5’-AGAUACUGCUAAGCAACGTTT-3’) and antisense siRNA strand, 5’-CCGUAUCGUAAAGCAGACUT-T-3’) were obtained from Ambion.

**Western Blot Analysis**—Total protein extracts were done by homogenizing HEK 293 cells and neurons in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 30 μM MG132, and protease inhibitor mixture (Complete; Roche Applied Science). Blots were probed with antibodies against ubiquitin-1 (13), anti-α-synuclein (BD Biosciences), mouse anti-HA (Covance), rabbit anti-HA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-actin (Santa Cruz), and mouse anti-HA (Covance), mouse anti-Myc (Santa Cruz) as described (19). Immunoprecipitates were washed with lysis buffer containing 500 mM NaCl and detected by Western blot.

**In Vitro Protein Kinase Assays**—HEK 293 cells were transfected with HA-synphilin-1 cDNAs. After 36 h of transfection, cells were lysed as in the in vitro ubiquitylation experiments. HA-synphilin-1 was immunoprecipitated with anti-HA antibody and washed in lysis buffer containing 500 mM NaCl. Immunoprecipitated synphilin-1 was incubated with recombinant GSK3β (New England Biolabs) at 37 °C for 1 h in buffer containing 40 mM Tris, pH 7.6, 2 mM dithiothreitol, 5 mM MgCl2, 2 μg/ml soybean trypsin inhibitor, 0.5 mM unlabeled ATP, 0.25 mM/μl [γ-32P]ATP. Reactions were ended with sample buffer and analyzed by SDS-PAGE using 8% gel. The amount of 32P-labeled synphilin-1 was quantified by PhosphorImager analysis. Equal loading of immunoprecipitated HA-synphilin-1 was determined by Western blot or Coomassie Blue staining.

**In Vitro Ubiquitylation Assays**—Synphilin-1 was translated using TNT wheat germ in vitro translation kit from Promega using [35S]methionine (Amersham Biosciences). In vitro translated proteins were incubated in reaction medium containing 40 mM Tris (pH 7.6), 5 mM MgCl2, 2 mM dithiothreitol, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 7.5 μg of ubiquitin, 1 μg of ubiquitin-activating enzyme, and 200 ng of UbcH5b. Reactions were incubated at 37 °C for 1 h and analyzed by SDS-PAGE. 35S-Labeled synphilin-1 was determined by PhosphorImager analysis.

For the in vitro ubiquitylation assays of prephosphorylated synphilin-1, HA-synphilin-1 was co-transfected with Myc-GSK3β into HEK 293 cells. After 36 h, cells were lysed by sonication in buffer containing 50 mM Tris-HCl (pH 7.4), 140 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM NaF, 2 mM Na3VO4, 30 μM MG132, and protease inhibitor mixture (Complete; Roche Applied Science). Immunoprecipitation of HA-synphilin-1 was carried out with anti-HA antibody for 4 h at 4 °C. Beads were washed with lysis buffer supplemented with 500 mM NaCl. Immunoprecipitated HA-synphilin-1 was incubated with the same ubiquitylation reaction medium used for the in vitro translated synphilin-1. Reactions were incubated at 37 °C for 1 h, and ubiquitylated synphilin-1 was detected by Western blot. Very harsh lysis conditions were used in order to prevent co-immunoprecipitation of GSK3β with synphilin-1.

**In Vivo Ubiquitylation Assays**—Transfected HEK 293 cells were incubated with 10 μM lactacystin for 12 h and then lysed in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM NaF, 2 mM Na3VO4, 10 μM lactacystin, and protease inhibitor mixture (Complete; Roche Applied Science). Immunoprecipitates were washed with lysis buffer containing 500 mM NaCl, and ubiquitylated synphilin-1 was detected by Western blot.

**Pulse-Chase Experiments**—Transfected HEK 293 cells were washed, incubated with methionine/cysteine-free medium for 1 h, pulsed with methionine/cysteine-free medium containing 200 μCi/ml [35S]methionine/cysteine (PerkinElmer Life Sciences) for 3 h, and subsequently chased in normal medium for the times specified. Cells were harvested, and HA-synphilin-1 immunoprecipitation was carried out as described above for the in vivo ubiquitylation assays. Immunoprecipitates were resolved on 8% SDS-polyacrylamide gels, and the amount of 35S-labeled synphilin-1 was quantified by PhosphorImager analysis.

**Immunocytochemistry Assays**—Transfected HEK 293 cells were treated for 8 h with 10 μM lactacystin, fixed with 4% paraformaldehyde for 15 min, and blocked in phosphate-buffered saline containing 0.2% Triton X-100 and 5% normal goat serum. Cells were stained with anti-HA (Covance) and anti-Myc (Santa Cruz) as described (19). Immunolabeling was detected using fluorescein isothiocyanate- and Cy3-labeled secondary antibodies (Jackson Laboratories). The percentage of cells containing cytosolic inclusions was counted by an investigator unaware of the treatment groups. Statistics of the number of inclusion-containing cells was analyzed by analysis of variance followed by Tukey’s post-test and by paired t test, when appropriate.
we sought to investigate whether synphilin-1 is a target of GSK3β phosphorylation of full-length synphilin-1 (Fig. 1).

Phosphorylation of full-length synphilin-1 in HEK 293 cells was significant by GSK3β. HEK 293 cells were co-transfected with full-length wild-type HA-synphilin-1 and siRNA to GSK3β and incubated with [32P]orthophosphate. Full-length HA-synphilin-1 was immunoprecipitated with an anti-HA antibody, and phosphorylated synphilin-1 was visualized by autoradiography. The lower panel shows equal immunoprecipitation of HA-synphilin-1 by Western blot using an anti-HA antibody, but not with control siRNA. Western blot was carried out using anti-GSK3β antibody. The fourth panel shows loading control of protein extracts carried out by incubating membrane with anti-actin antibody.

To confirm that synphilin-1 is phosphorylated by GSK3β, we carried out in vivo phosphorylation assays in which HEK 293 cells were co-transfected with full-length HA-synphilin-1 and increasing amounts of Myc-GSK3β and [γ-32P]ATP. Phosphorylated synphilin-1 was visualized by autoradiography. The lower panel shows equal immunoprecipitation of HA-synphilin-1 by Western blot using an anti-HA antibody.

Results

Phosphorylation by GSK3β—We found that synphilin-1 has 22 putative GSK3β phosphorylation sites according to the motif X(S/T)XXS. Thus, we sought to investigate whether synphilin-1 is a target of GSK3β in vivo. Phosphorylation of full-length synphilin-1 in HEK 293 cells was significantly inhibited by two selective GSK3β inhibitors, SB415286 and kenpaullone (23) (Fig. 1A). Lithium chloride, another GSK3β inhibitor, also inhibited the phosphorylation of full-length synphilin-1 (data not shown).

In order to further verify the specificity of synphilin-1 phosphorylation by GSK3β, we carried out in vitro phosphorylation experiments using siRNA to suppress GSK3β expression. HEK 293 cells were transfected with full-length HA-synphilin-1 and siRNA to GSK3β or negative control siRNA. The siRNA to GSK3β, but not the control siRNA, was effective in decreasing the expression of GSK3β by at least 90% (Fig. 1B). We found that siRNA to GSK3 significantly decreased the phosphorylation of full-length synphilin-1 (Fig. 1B).

To confirm that synphilin-1 is phosphorylated by GSK3β, we carried out in vivo phosphorylation assays in which HEK 293 cells were co-transfected with full-length HA-synphilin-1 and increasing amounts of Myc-GSK3β. Accordingly, GSK3β increased synphilin-1 phosphorylation in a dose-dependent manner (Fig. 1C). In addition, the constitutively active GSK3β S9A mutant (24) phosphorylated synphilin-1 more efficiently than wild-type GSK3β (Fig. 1D). In vitro phosphorylation experiments with immunoprecipitated full-length synphilin-1 and recombinant GSK3β confirmed that synphilin-1 is phosphorylated by GSK3β, indicating that the GSK3β effect is direct (Fig. 1E).

The ability of GSK3β to interact with synphilin-1 was also investigated by co-immunoprecipitation experiments using HEK 293 cells co-transfected with HA-synphilin-1 and Myc-GSK3β. When anti-HA was used to immunoprecipitate HA-synphilin-1, Myc-GSK3β was found associated with HA-synphilin-1 (Fig. 2). The interaction of GSK3β with synphilin-1 is specific, since the control Myc-FKBP12 did not co-immunoprecipitate with HA-synphilin-1 (Fig. 2). To map the GSK3β-binding domain of synphilin-1, we co-transfected different HA-tagged synphilin-1 fragments into HEK 293 cells with Myc-GSK3β and carried out co-immunoprecipitation experiments. Different regions of synphilin-1 interacted with GSK3β, and a higher co-immunoprecipitation of GSK3β was observed with the synphilin-1 region containing amino acids 550–769 (Fig. 2).

GSK3β activity is strongly stimulated by 14–3–3 protein, which also interacts with α-synuclein and accumulates in Lewy bodies (25–27).
GSK3β Decreases Synphilin-1 Ubiquitylation Promoted by SIAH

To investigate whether phosphorylation of synphilin-1 ubiquitylation promoted by SIAH-2 was significantly inhibited by the addition of casein kinase II, GSK3β, and phosphorylated synphilin-1 was detected by autoradiography. The lower panel shows equal immunoprecipitation of HA-synphilin-1 by Coomassie Blue staining.

FIGURE 3. Protein 14-3-3 co-immunoprecipitates with synphilin-1 and enhances synphilin-1 phosphorylation by GSK3β. A, HEK 293 cells were co-transfected with HA-synphilin-1, Myc-GSK3β, Myc-14-3-3, or Myc-FKBP12 as control. HA-synphilin-1 was immunoprecipitated (IP HA) using an anti-HA antibody, and samples were subjected to Western blot analysis using an anti-Myc antibody to check for Myc-GSK3β co-immunoprecipitation (middle panel). The upper panel shows total expression of Myc-GSK3β, Myc-14-3-3, and Myc-FKBP12 in the HEK 293 cells determined by Western blot using an anti-Myc antibody. The lower panel shows the amount of immunoprecipitated full-length HA-synphilin-1 by Western blot using an anti-HA antibody. B, HEK 293 cells were co-transfected with HA-synphilin-1, Myc-GSK3β, and Myc-14-3-3. Cells were incubated with [32P]orthophosphate, HA-synphilin-1 was immunoprecipitated with an anti-HA antibody, and phosphorylated synphilin-1 was detected by autoradiography. The lower panel shows equal immunoprecipitation of HA-synphilin-1 by Coomassie Blue staining.

FIGURE 2. GSK3β co-immunoprecipitates with synphilin-1. HEK 293 cells were co-transfected with HA-synphilin-1 (full-length or different fragments) and Myc-GSK3β or Myc-FKBP12 as control. HA-synphilin-1 was immunoprecipitated (IP HA) using an anti-HA antibody, and samples were subjected to Western blot analysis using an anti-Myc antibody to check for Myc-GSK3β co-immunoprecipitation (middle panel). The upper panel shows equal expression of Myc-GSK3β and Myc-FKBP12 in the HEK 293 cells determined by Western blot using an anti-Myc antibody. The lower panel shows the amount of immunoprecipitated HA-synphilin-1 (full-length or different fragments) by Western blot using an anti-HA antibody.

Conceivably, the effect of GSK3β on synphilin-1 ubiquitylation could be due to interference with the components of the ubiquitin system or with a change of SIAH activity. However, the catalytic activity of SIAH-1 monitored by its autoubiquitylation was not affected by GSK3β (Fig. 4B). Moreover, SIAH-1 does not possess consensus sequences for GSK3β phosphorylation.

In order to confirm a direct effect of GSK3β on synphilin-1 ubiquitylation, we carried out in vitro ubiquitylation experiments using synphilin-1 prephosphorylated by GSK3β. For this, HEK 293 cells were co-transfected with HA-synphilin-1 and Myc-GSK3β and lysed under harsh conditions to avoid co-immunoprecipitation of GSK3β. Also, to avoid dephosphorylation of synphilin-1, all of the immunoprecipitation steps were carried out in the presence of phosphatase inhibitors. We found that the ubiquitylation of prephosphorylated synphilin-1 by GSK3β was significantly smaller than that observed with synphilin-1 immunoprecipitated from cells co-transfected with the control protein FKBP12 (Fig. 4C). This indicates that the effect of GSK3β on the ubiquitylation of synphilin-1 is due to a direct effect on synphilin-1 and not on the components of the ubiquitination system. The lack of Myc-GSK3β co-immunoprecipitation with HA-synphilin-1 was ascertained by reprobing the immunoprecipitation membrane with an anti-Myc antibody (Fig. 4C).

We next examined if phosphorylation by GSK3β interferes with the in vivo ubiquitylation of synphilin-1. For this, ubiquitylation of synphilin-1 by FLAG-ubiquitin was detected by immunoprecipitating synphilin-1 by FLAG-ubiquitin. The results showed that the ubiquitylation of synphilin-1 in vivo was significantly decreased by GSK3β expression. Cells co-transfected with SIAH-1 had higher levels of ubiquitylated synphilin-1, which were also significantly augmented by SB415286 treatment (Fig. 4D). Overexpression of GSK3β decreased synphilin-1 ubiquitylation in SIAH-1-transfected cells, confirming the
**FIGURE 4. Effect of GSK3β in the ubiquitylation of synphilin-1 by SIAH.** A, GSK3β decreases in vitro synphilin-1 ubiquitylation by SIAH. In vitro translated synphilin-1 was incubated with the indicated components of the ubiquitin system, in the presence of glutathione S-transferase (GST)-SIAH-2, GSK3β, and CKII, and ubiquitylated synphilin-1 was visualized by autoradiography. B, ubiquitin-activating enzyme, B, autoubiquitylation of SIAH-1 is not affected by GSK3β. In vitro translated SIAH-1 was incubated with ubiquitin system components in the presence or absence of GSK3β, and ubiquitylated SIAH-1 was visualized by autoradiography. C, GSK3β decreases in vitro ubiquitylation of prephosphorylated synphilin-1. HEK 293 cells were transfected with HA-synphilin-1 and Myc-GSK3β for the control Myc-FKBP12. HA-immunoprecipitates (IP HA) were incubated with the indicated components of the ubiquitin system and glutathione S-transferase-SIAH-2, and ubiquitylation of HA-synphilin-1 was determined by Western blot using purified anti-synphilin-1 antibody (middle panel). The upper panel shows the expression of Myc-GSK3β in the transfected HEK 293 cells determined by Western blot using an anti-Myc antibody. The lower panel shows the lack of residual Myc-GSK3β in the synphilin-1 immunoprecipitate due to the harsh immunoprecipitation conditions, as described under “Experimental Procedures.” D, GSK3β decreases the in vitro ubiquitylation of synphilin-1 by SIAH. Transfected HEK 293 cells were treated for 12 h with 10 μM lactacystin. HA-synphilin-1 was immunoprecipitated using an anti-HA antibody. Immunoprecipitates were analyzed for ubiquitylation using an anti-FLAG antibody (middle panel). The middle panel (far right) shows a less exposed picture of ubiquitylated HA-synphilin-1 in the presence of SIAH-1 (underlined last three lanes). The upper panel shows an equal presence of FLAG-ubiquitylated proteins in the HEK 293 cells determined by Western blot using an anti-FLAG antibody. The lower panel shows the amount of immunoprecipitated HA-synphilin-1 by Western blot using an anti-HA antibody.

We found that GSK3β consistently increased synphilin-1 steady-state levels and that this was more prominent in SIAH-1-transfected cells (Fig. 5A). To test whether GSK3β modulates synphilin-1 half-life, we carried out pulse-chase experiments in SIAH-1-transfected cells. In cells pulsed with [35S]methionine, GSK3β promoted a significant increase in synphilin-1 half-life, indicating that GSK3β decreases the degradation of synphilin-1 promoted by SIAH-1 (Fig. 5B). Conversely, inhibition of GSK3β by SB415286 accelerated the rate of synphilin-1 degradation (Fig. 5B). This indicates that GSK3β alters ubiquitin-dependent degradation of synphilin-1 and that its effects on steady-state synphilin-1 levels are not due to changes in transcription.

The effects of GSK3β in the ubiquitylation and degradation of synphilin-1 are not due to decreased interaction between synphilin-1 and SIAH. Neither His-GSK3β nor GSK3β inhibitor SB415286 affected the co-immunoprecipitation of HA-synphilin-1 with Myc-SIAH-1 (data not shown). The casein kinase II inhibitor DRB, previously shown to decrease synphilin-1/α-synuclein binding (28), had no effect on synphilin-1 ubiquitylation, indicating that SIAH is not affected by GSK3β.
GSK3β Decreases Synphilin-1 Ubiquitylation Promoted by SIAH

In order to determine whether GSK3β phosphorylation also modulates endogenous levels of synphilin-1 in neuronal cells, we incubated primary cortical neuronal cultures in the presence of SB415286. Inhibition of GSK3β by SB415286 significantly reduced synphilin-1 steady-state levels in cortical neuronal cells (Fig. 6). The proteasomal inhibitor MG132 completely restored synphilin-1 levels, indicating that GSK3β is a physiological modulator of endogenous neuronal synphilin-1 degradation by the proteasome system.

Identification of GSK3β Phosphorylation Site—To identify the GSK3β phosphorylation sites among the 22 putative sites throughout the protein, we first mapped the region in synphilin-1 that is preferentially phosphorylated. We carried out in vivo phosphorylation experiments using HEK 293 cells transfected with HA-synphilin-1 fragments that together encompass the whole synphilin-1 open reading frame. We found that synphilin-1 is preferentially phosphorylated in the region encoding amino acids 550–769 (Fig. 7A). Note that this region displayed a more significant binding to GSK3β than other synphilin-1 regions (Fig. 2).

Further breakdown of this amino stretch into two fragments (amino acids 550–659 and 660–769) showed that the region between amino acids 550 and 659 is responsible for an important portion of endogenous synphilin-1 phosphorylation (Fig. 7B). Quantitative analysis of the amount of incorporated phosphate (Fig. 7B, upper panel) relative to the amount of immunoprecipitated synphilin-1 fragment measured by densitometry (Fig. 7B, lower panel) showed that the phosphorylation of amino acids 550–659 of synphilin-1 was ~3 fold larger than the phosphorylation of synphilin-1 region encoding amino acids 660–769 (Fig. 7B). Notice that synphilin-1 550–659 appeared as a phosphorylated doublet, compatible with different degrees of phosphorylation that affect the mobility of the fragment.

We next examined the protein kinases involved in the in vivo phosphorylation of the amino acid stretch 550–659 of synphilin-1. Among the several protein kinase inhibitors utilized, only the GSK3β inhibitors, SB415286 and kenpaullone, were able to robustly inhibit the phosphorylation of the synphilin-1 550–659 fragment (Fig. 7C). Protein kinase C inhibitor (Bis I) inhibited synphilin-1 550–659 phosphorylation to a lesser degree. Inhibitors of casein kinase II (DRB), tyrosine kinase (genistein), protein kinase A (H-89), and phosphatidylinositol 3-kinase (wortmannin) had no effect in the phosphorylation of the synphilin-1 550–659 fragment (Fig. 7C and data not shown).

To further confirm the specificity of synphilin-1 550–659 phosphorylation by GSK3β, we carried out in vivo phosphorylation experiments using siRNA to suppress GSK3β expression. HEK 293 cells were trans-
fected with HA-synphilin-1 550–659 and siRNA to GSK3β or control siRNA. Phosphorylation analysis of immunoprecipitated HA-synphilin-1 550–659 revealed that the siRNA to GSK3β, but not the control siRNA, promoted the disappearance of the synphilin-1 phosphorylated upper band, indicating that GSK3β specifically phosphorylates synphilin-1 within amino acids 550–659 (Fig. 7D). As in Fig. 1B, the siRNA to GSK3β was effective in decreasing the expression of GSK3β by more than 90% (Fig. 7D).

We next investigated whether the synphilin-1 region encoding amino acids 660–769 could also be phosphorylated by GSK3β in vivo. HEK 293 cells were transfected with HA-synphilin-1 660–769 and treated with the GSK3β inhibitors, SB415285 and kenpaullone. Phosphorylation analysis of immunoprecipitated HA-synphilin-1 660–769 revealed that neither SB415285 nor kenpaullone inhibited the phosphorylation of the synphilin-1 660–769 fragment (Fig. 7E), indicating that GSK3β does not phosphorylate synphilin-1 within amino acids 660–769.

We next sought to identify the residues within amino acids 549–679 of synphilin-1 that are phosphorylated by GSK3β. We generated a series of full-length HA-synphilin-1 constructs with point mutations of the nine putative GSK3β sites within this region. We found that substitution of Ser556 to Ala decreased the phosphorylation level of full-length synphilin-1 as revealed by in vitro assay using immunopurified synphilin-1.
GSK3β Decreases Synphilin-1 Ubiquitylation Promoted by SIAH

Control siRNA did not change the formation of synphilin-1-SIAH inclusions (Fig. 9, C and D), indicating that GSK3β specifically modulates synphilin-1 inclusion formation.

To further determine the importance of GSK3β for synphilin-1 degradation and inclusion formation, we carried out experiments with synphilin-1 S556A mutant that display deficient GSK3β phosphorylation (Fig. 8). S556A mutant elicited inclusions in a larger number of cells (40% increase) when compared with wild-type synphilin-1 (Fig. 9B). It is noteworthy that the increase in inclusion formation by the S556A mutant is not due to a change in synphilin-1 half-life. Pulse-chase experiments showed that S556A mutant displayed the same half-life as wild-type synphilin-1 (data not shown).

Synphilin-1 was recently shown to inhibit proteasomal function in vivo, but regulation of this process is unknown (18). A GFPu accumulation assay has been shown to reflect proteasomal impairment (30). Using this assay, we sought to determine whether synphilin-1 phosphorylation by GSK3β also alters the ability of synphilin to inhibit the proteasome. Since GSK3β possesses numerous targets in addition to synphilin-1, experiments testing GSK3β inhibitor effect on proteasomal function will obviously not be informative regarding the role of synphilin. Thus, we took advantage of the phosphorylation-deficient S556A mutant that increases synphilin-1-SIAH inclusions in order to study the role of GSK3β phosphorylation at this site. We found that S556A mutant was more effective than wild-type synphilin-1 in promoting accumulation of GFPu (Fig. 10). This implies that inhibition of the proteasome by synphilin-1 is also modulated by GSK3β phosphorylation.

FIGURE 8. Identification of Ser556 as a major GSK3β phosphorylation site. A, full-length HA-synphilin-1 (wild type or mutants) were immunoprecipitated from transfected HEK 293 cells and incubated with recombinant GSK3β and [γ-32P]ATP. Phosphorylated HA-synphilin-1 was detected by autoradiography. The lower panel shows immunoprecipitation of HA-synphilin-1 by Coomassie Blue staining. B, S556A decreased phosphorylation of synphilin-1, HEK 293 cells were co-transfected with full-length HA-synphilin-1 (wild type or mutants) and Myc-GSK3β and incubated with [γ-32P]orthophosphate. HA-synphilin-1 was immunoprecipitated with an anti-HA and detected by autoradiography. The lower panel shows immunoprecipitation of HA-synphilin-1 by Coomassie Blue staining.

DISCUSSION

We have previously shown that synphilin-1 is ubiquitylated by SIAH proteins and that ubiquitylated synphilin-1 is prone to aggregate into inclusions (19). The present paper describes a mechanism that regulates synphilin-1 ubiquitylation with implications for inclusion body formation. In a preliminary report, Wakabayashi and co-workers showed phosphorylation of synphilin-1 by GSK3β in vitro, but the function and possible occurrence of in vivo phosphorylation was not explored (31). Using pharmacological and siRNA approaches, we have now demonstrated that synphilin-1 is phosphorylated in vivo by GSK3β.

Phosphorylation of synphilin-1 by GSK3β decreased ubiquitylation and degradation of synphilin-1 promoted by SIAH. We show that GSK3β activity modulates the endogenous levels of synphilin-1 in neurons, indicating that GSK3β physiologically regulates synphilin-1 levels. Inhibition of GSK3β promoted a robust increase in the ability of synphilin-1 to form inclusions in the presence of SIAH. Additionally, mutation in GSK3β phosphorylation site potentiates synphilin-1 ability to inhibit the proteasome.

Our strategy to first map the region of phosphorylation to the 550–659 fragment and then carry out site-directed mutagenesis allowed us to identify Ser556 as a major site for phosphorylation by GSK3β among 22 putative sites. Mutation of Ser556 significantly inhibited synphilin-1 phosphorylation in vitro and in vivo. Despite the evidence that GSK3β inhibitor modulates endogenous synphilin-1 levels and half-life, S556A mutation did not change synphilin-1 degradation promoted by SIAH. This indicates that other phosphorylation sites within synphilin-1 may be required to regulate its degradation rate, since the mutation did not completely abolish phosphorylation by GSK3β. The mutagenesis of additional phosphorylation sites may be necessary to fully reveal the role of phosphorylation in the ubiquitylation and degradation of synphilin-1. Nevertheless, the S556A mutant disclosed a new role for GSK3β phosphorylation in modulating synphilin-1 inclusions. The evidence that the
S556A mutant, GSK3β inhibitors, and siRNA to GSK3β increased inclusion body formation indicates that phosphorylation at Ser556 modulates intracellular synphilin-1 aggregates. We have previously shown that ubiquitylation of synphilin-1 is required for inclusion body formation elicited by SIAH (19). Our data now suggest that the modulation of inclusion body formation also depends on GSK3β phosphorylation.

Recently, Mouradian and co-workers found that casein kinase II phosphorylation at a still unidentified region of synphilin-1 is required for synphilin-1/synuclein aggregation (29). Under our experimental conditions, casein kinase II inhibitor had no effect on synphilin-1 ubiquitylation or its ability to form cytosolic aggregates with SIAH-1.

 Accumulation of abnormal or unfolded proteins leads to ER stress, which can ultimately lead to cell death (32). Several lines of evidence suggest that ER stress may occur in PD. The parkin substrate pael-r was shown to cause ER stress in dopaminergic neuroblastoma cells (33). 6-Hydroxydopamine and 1-methyl-4-phenylpyridinium were shown to activate ER stress-related proteins, and, more recently, GSK3β was shown to mediate 6-hydroxydopamine-induced neuronal death (34, 35). In addition, GSK3β is known to mediate cell death during ER stress and is associated with neurodegenerative diseases, including Alzheimer disease (36, 37). In this context, the decrease we found in neuronal synphilin-1 levels by GSK3β inhibition may have important pathological implications. Soluble synphilin-1 was shown to promote cellular toxicity, whereas synphilin-1 inclusions were shown to protect cells from dying (19–21). Our results suggest that inhibition of phosphorylation by GSK3β will decrease synphilin-1 levels and also favor its deposition into inclusions, implying that GSK3β may play a role in the ability of synphilin-1 to aggregate into Lewy bodies. On the other hand,
activation of GSK3β by ER stress and consequent increase of synphilin-1 levels might contribute to the neurotoxicity observed in PD.

The present study shows that 14-3-3 protein potentiates phosphorylation by GSK3β. Since 14-3-3 was previously shown to associate with α-synuclein (26), we raise the possibility that a 14-3-3 and GSK3β may be part of a macromolecular complex together with synphilin-1, α-synuclein, and SIAH. Further analysis of synphilin-1 phosphorylation status in the substantia nigra of patients with PD will help to determine the role of synphilin-1 in the death of dopaminergic neurons.

Inclusion bodies are induced by proteasome inhibitors (19, 38). α-Synuclein as well as synphilin-1 inhibits proteasomal activity (8, 18). Our study now demonstrated that the GSK3β-phosphorylation-deficient mutant is more effective than wild-type synphilin-1 in inhibiting proteasomal activity. Although the mechanism of proteasomal dysfunction is not known, our results imply that GSK3β phosphorylation of synphilin-1 may contribute to proteasomal dysfunction thought to occur in PD (12).

In summary, we showed that synphilin-1 is phosphorylated in vivo by GSK3β, causing a decrease in ubiquitin-dependent degradation of synphilin-1. Inhibition of GSK3β increased the formation of intracellular inclusions induced by proteasome inhibitors, suggesting that GSK3β activity may be relevant for Lewy body formation.

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