Effect of Ser-129 Phosphorylation on Interaction of α-Synuclein with Synaptic and Cellular Membranes

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Background: The majority of α-synuclein is phosphorylated at serine 129 in Lewy bodies. Pathological serine 129 phosphorylation regulates membrane accumulation of mutant α-synuclein. The relationship of serine 129 phosphorylation to pathogenic aggregation of normal and mutant α-synuclein may be governed by distinct effects on phosphoprotein membrane accumulation.

Results: The membrane association of PD-linked mutant α-synuclein, but not wild-type α-synuclein, was increased by serine 129 phosphorylation. 

Conclusion: Pathological serine 129 phosphorylation regulates membrane accumulation of mutant α-synuclein. The relationship of serine 129 phosphorylation to pathogenic aggregation of normal and mutant α-synuclein may be governed by distinct effects on phosphoprotein membrane accumulation.

In the healthy brain, less than 5% of α-synuclein (α-syn) is phosphorylated at serine 129 (Ser(P)-129). However, within Parkinson disease (PD) Lewy bodies, 89% of α-syn is Ser(P)-129. The effects of Ser(P)-129 modification on α-syn distribution and solubility are poorly understood. As α-syn normally exists in both membrane-bound and cytosolic compartments, we examined the binding and dissociation of Ser(P)-129 α-syn and analyzed the effects of manipulating Ser(P)-129 levels on α-syn membrane interactions using synaptosomal membranes and neural precursor cells from α-syn-deficient mice or transgenic mice expressing human Parkinson disease (PD) Lewy bodies, 89% of α-syn is Ser(P)-129. We first evaluated the recovery of the Ser(P)-129 epitope following either α-syn membrane binding or dissociation. We demonstrate a rapid turnover of Ser(P)-129 during both binding to and dissociation from synaptosomal membranes. Although the membrane binding of WT α-syn was insensitive to modulation of Ser(P)-129 levels by multiple strategies (the use of phosphomimic S129D and nonphosphorylated S129A α-syn mutants; by enzymatic dephosphorylation of Ser(P)-129 or proteasome inhibitor-induced elevation in Ser(P)-129; or by inhibition or stable overexpression of PLK2), PD mutant Ser(P)-129 α-syn showed a preferential membrane association compared with WT Ser(P)-129 α-syn. Collectively, these data suggest that phosphorylation at Ser-129 is dynamic and that the subcellular distribution of α-syn bearing PD-linked mutations, A30P or A53T, is influenced by the phosphorylation state of Ser-129.

Parkinson disease (PD) is a progressive neurodegenerative disorder characterized by a loss of dopaminergic neurons in the substantia nigra pars compacta and depletion of dopamine in the striatum. The major pathological hallmark in affected brains is the presence of α-synuclein (α-syn)-positive Lewy bodies (LBs) in surviving neurons. Normally, α-syn is an abundant presynaptic protein thought to play a role in synaptic plasticity (1, 2). However, multiplication of the gene encoding α-syn or missense mutations (A53T, A30P, or E46K) are known to cause inherited forms of PD (3). In vitro studies suggest that α-syn is natively unfolded in aqueous solution and that exposure to lipids stabilizes the amino terminus in an amphipathic α-helix that aligns polar and nonpolar residues into opposing orientations (4–6). This secondary structure confers the lipid-binding properties for direct membrane interaction such that purified recombinant α-syn can bind to small diameter artificial vesicles rich in acidic phospholipids (7, 8), to purified synaptic vesicles and membranes (9–11), and to membranes within intact cells (12).

α-syn also has the propensity to aggregate and can form amyloid-like fibrils, which are the main component of LBs (13). Increasing evidence suggests that phosphorylation may be an important regulator of α-syn oligomerization, fibrillogenesis, LB formation, and neurotoxicity in vivo (14). Immunohistochemical and biochemical studies suggest that the majority of α-syn within LBs from patients with PD and related synucleinopathies is phosphorylated at serine residue 129 (Ser(P)-129) (15–18). Moreover, alteration of Ser-129 to the negatively charged amino acid aspartate, to mimic phosphorylation, significantly enhanced α-syn toxicity in Drosophila, although its toxicity in studies with rodents has been equivocal (14, 19, 20). Several kinases, including casein kinases 1 and 2 (21–23) and members of the G-protein-coupled receptor kinase family (24),
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are capable of phosphorylating α-syn at Ser-129. However, recent evidence suggests that a member of the polo-like kinase family, PLK2, is the prime contributor to phosphorylation of α-syn at Ser-129 (25, 26). Inhibitory RNA-induced knockdown of PLK2 in human cortical cultures or the knock-out of the plk2 gene in mice reduced the levels of Ser(P)-129 α-syn by >70%, indicating that PLK2 is responsible for the majority of phosphorylation of α-syn at Ser-129 in vivo. Nevertheless, Ser-129 phosphorylation reduced α-syn aggregation in vitro (27), and pharmacological inhibition of kinases associated with phosphorylation of Ser-129 failed to reduce α-syn aggregation in a cellular model (28), suggesting that α-syn aggregation is either inhibited or is independent of Ser-129 phosphorylation.

Because inclusion formation may be linked to nucleation-elongation process initiated on membranes (29), we hypothesized that phosphorylation of α-syn at Ser-129 may affect membrane binding. Therefore, we took advantage of in vitro binding and dissociation assays (11, 30) to analyze the effect of phosphorylation at Ser-129 on the interaction of α-syn with synaptic membranes. We then tested the effects of manipulating the level of Ser-129 phosphorylation, both pharmacologically and by virus-mediated expression of PLK2, on membrane binding of α-syn in neural precursor cells prepared from transgenic mice expressing human α-syn.

EXPERIMENTAL PROCEDURES

Synaptosomal Membrane Preparation

Synaptosomal membranes were prepared as described previously (31). Briefly, murine cerebral cortices were homogenized in ice-cold buffer A (320 mM sucrose, 1 mM EGTA, and 5 mM HEPES (pH 7.4)) and centrifuged (1000 × g, 10 min). The supernatant was centrifuged (24,000 × g, 10 min), and the resulting pellet (P2) was resuspended in buffer A. The P2 fraction was loaded onto a discontinuous Ficoll gradient (13, 9, and 5% in buffer A) and centrifuged (35,000 × g, 35 min). The 13 to 9% interface, containing intact synaptosomes, was resuspended in buffer B (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 mM NaHCO3, 1.2 mM Na2HPO4, 1 mM MgCl2, 1 mM EGTA, and 10 mM glucose) and centrifuged (24,000 × g, 10 min). Synaptosomes were lysed in buffer C (10 mM HEPES, 18 mM KCl (pH 7.2)), centrifuged (24,000 × g, 10 min), and resuspended in buffer D (25 mM HEPES, 125 mM KOAc, and 2.5 mM MgCl2).

Cytosol Preparation

Brains from α-syn KO mice and mice overexpressing WT, A30P, or A53T α-syn were homogenized in 85 mM sucrose, 100 mM KOAc, 1 mM MgOAc, and 20 mM HEPES (pH 7.4). The homogenate was centrifuged (15,000 × g, 10 min), and the resulting supernatant was centrifuged (100,000 × g, 1 h). The supernatant was dialyzed for 4 h in 145 mM KOAc and 25 mM HEPES (pH 7.2). Protein concentration was determined by BCA protein assay (Pierce).

Binding/Dissociation Experiments

Synaptosomal membranes were incubated with cytosol for 10 min at 37 °C in the absence or presence of a serine/threonine phosphatase inhibitor mixture (phosphatase inhibitor mixture 3 purchased from Sigma) and 1 μM okadaic acid. Membrane and supernatant were separated by centrifugation (24,000 × g, 10 min), and α-syn levels in the membrane and supernatant fractions were quantified by Western blotting.

Western Blotting

Proteins were separated by electrophoresis using 12% Tris-glycine polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Life Sciences) and probed with antibodies against α-syn (Syn-1, BD Transduction Laboratories, 1:1000), Ser(P)-129 α-syn (clone 11A5, 1:500, Elan Pharmaceuticals, GAPDH (Sigma 1:1000), syntaxin (Sigma, 1:1000), tetra-His (Qiagen, 1:1000), and ubiquitin (Dako, 1:1000). Bound HRP-conjugated anti-mouse or anti-rabbit IgG (Sigma) was revealed by chemiluminescence using ECL Plus (GE Healthcare) and quantified with a Storm 860 fluorescent imager and ImageQuant software (GE Healthcare).

Isolation of Mouse Whole Brain Neural Precursor Cells

Mouse whole brain neural precursor cells (NPC) were prepared according to the method of Ray and Gage (32). Briefly, the brains of six mice, overexpressing human WT, A30P, or A53T α-syn under the control of the hamster prion protein promoter (see Ref. 30) were chopped into pieces (~2 mm3) before being incubated at 37 °C in 10 volumes (g/ml) of DMEM containing papain (2.5 units/ml), protease type 1 (1 unit/ml), and DNase I (250 units/ml). The tissue was triturated every 5 min until a smooth consistency was achieved (~30 min). Following digestion, the cell suspension was mixed with an equal volume of DMEM/F-12/N2 containing 10% FBS. The suspension was then passed through a sterilized 15-μm nylon mesh filter and centrifuged (1000 × g for 3 min). The pellet was resuspended in DMEM/F-12/N2 containing 10% FBS and mixed with an equal volume of Percoll (9:1 v/v Percoll/PBS) and centrifuged (20,000 × g for 30 min at 18 °C). NPC were harvested from the low buoyancy fraction (~5 ml above the red blood cell layer). Cells were washed in cold PBS containing 1× antibiotic/antimycotic solution (containing penicillin, streptomycin, and amphotericin B) before plating in DMEM/F-12/N2 containing 1× antibiotic/antimycotic solution, βFGF (20 mg/ml), EGF (20 ng/ml), and heparin (5 μg/ml). Proliferating NPC began to grow in clusters ~2–3 weeks post-isolation. During this period, half the media were changed weekly, and βFGF (20 mg/ml), EGF (20 ng/ml), and heparin (5 mg/ml) were added every 3–4 days.

Tissue Culture

SHSY5Y Cells—SHSY5Y cells (ATCC) were maintained under an atmosphere of air and 5% CO2 at 37 °C. The medium consisted of DMEM containing 4.5 g/liter glucose, 0.01 mM sodium pyruvate, 1 μM nonessential amino acids, and 10% fetal bovine serum (Multitcell). Cells were cultured in 10-cm plates and passaged at ~75% confluence.

Maintenance of NPC—NPC were maintained under an atmosphere of air and 5% CO2 at 37 °C. The medium consisted of DMEM/F-12/N2 (Invitrogen) containing βFGF (20 mg/ml, Invitrogen), EGF (20 ng/ml, Invitrogen), and heparin (5 mg/ml, Invitrogen).
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Cells were fixed with 4% formaldehyde for 15 min, permeabilized with 0.3% Triton X-100, blocked in 100% HHG (1 mM HEPES, 2% horse serum, 10% goat serum in Hanks’ buffered salt solution), and incubated with primary antibodies (11A5 1:500 and anti-His 1:500) overnight at 4 °C. Cells were then incubated with fluorescent secondary antibodies (Alexa Fluor 488 or 555 1:500) for 1 h at room temperature before mounting with ProLong® Gold DAPI mounting media (Invitrogen).

Statistical Analysis

Statistical comparisons were carried out using GraphPad Prizm version 4.0 for Windows. In all cases either a one-way or two-way ANOVA, followed by a Bonferroni post hoc multiple comparison test, was used.

RESULTS

Total α-syn and Ser(P)-129 α-syn in Synaptosomes—The distribution of total α-syn and Ser(P)-129 α-syn was examined in synaptosomes prepared from transgenic mice expressing human (WT, A30P, or A53T) but not endogenous murine α-syn (Fig. 1). The total α-syn expression relative to GAPDH was similar between the WT, A30P, and A53T isoforms. However, total A30P Ser(P)-129 α-syn was lower than the corresponding levels of both WT and A53T Ser(P)-129 α-syn. A30P Ser(P)-129 α-syn was reduced in both the membrane-bound and cytosolic fractions. Thus, similar to unphosphorylated α-syn, Ser(P)-129 α-syn can also exist as both membrane-bound and cytosolic forms.

Does Phosphorylation at Ser-129 Regulate Binding or Dissociation of α-syn to/from Synaptic Membranes?—The effect of Ser-129 phosphorylation on α-syn membrane interaction is poorly understood, and despite the small proportion of Ser(P)-129 α-syn in normal brains, it remains possible that Ser(P)-129 represents an intermediate step for α-syn membrane association or dissociation. Therefore, to determine whether phosphorylation or dephosphorylation at Ser-129 may play a role in regulating α-syn exchange between membrane and cytosolic compartments, we measured the amount of Ser(P)-129 α-syn before and after membrane binding or dissociation in vitro as described previously (11, 30).

Binding of α-syn to synaptic membranes was determined by incubating α-syn−/− synaptic membranes with brain cytosol prepared from mice overexpressing the human form of α-syn (WT, A30P, or A53T) (11). The recovery of total α-syn and Ser(P)-129 α-syn in the membrane and cytosolic fractions was compared with the starting material. Following incubation with WT α-syn cytosol, 30 ± 3% of total α-syn was recovered in the membrane fraction, and 67 ± 2% remained in the cytosol (Fig. 2A). Using cytosol prepared from A30P or A53T mice, slightly lower amounts, 10 ± 4 and 12 ± 5% respectively, were recovered on membranes, and the corresponding 90 ± 2 and 88 ± 3% remained in the cytosol (Fig. 2, B and C). Therefore, recovered
α-syn matched the quantity of α-syn in the starting material. Surprisingly, however, the net recovery of Ser(P)-129 α-syn in the same experiments was substantially less than the total input amount. Only 3 ± 1 and 63 ± 2% of Ser(P)-129 WT α-syn were recovered in the membrane and cytosolic fractions, respectively (Fig. 2D), totaling 66% of initial Ser(P)-129 WT α-syn, and suggesting a loss of 34% of Ser(P)-129 WT α-syn. Similarly, net recovery of both A30P and A53T Ser(P)-129 α-syn was also reduced after binding. Cytosolic Ser(P)-129 α-syn was 55 ± 5 and 65 ± 5%, respectively, and the level recovered on the membrane was only 10 ± 3 and 4 ± 1% of the starting input amounts (Fig. 2E and F). These data suggest net loss of phosphorylated α-syn during the process of binding with the synaptic membrane.

Using another cell-free assay to measure α-syn movement off membranes (30), we then investigated changes to Ser(P)-129 α-syn during dissociation from synaptic membranes prepared from mice overexpressing WT, A30P, or A53T α-syn into cytosol prepared from α-syn−/− mice. As illustrated in Fig. 3A, following incubation with membranes from WT mice, 7 ± 1% of α-syn dissociated to the cytosol and 92 ± 2% remained membrane-bound. A similar pattern was observed with membranes prepared from both A30P and A53T mice, with 10 ± 2 and 12 ± 1% found in the cytosol and 88 ± 2 and 88 ± 2% remaining membrane-bound, respectively (Fig. 3, B and C). Thus, following incubation with α-syn−/− cytosol, a small percentage of WT, A30P, and A53T α-syn disassociates into the cytosol, with the majority remaining membrane-bound. We then measured the disassociation of Ser(P)-129 α-syn from synaptic membranes using the same assay. As illustrated in Fig. 3D, following exposure to α-syn−/− cytosol the level of membrane-bound Ser(P)-129 α-syn was reduced to 52 ± 4% of starting material. However, only 2 ± 1% of WT Ser(P)-129 α-syn was found in the cytosol. Thus, ~46% of Ser(P)-129 α-syn was lost during the incubation. A similar loss of Ser(P)-129 was observed with both A30P and A53T α-syn with membrane-bound Ser(P)-129 α-syn reduced to 40 ± 4 and 45 ± 6%, respectively, and the amount recovered in the cytosol was only 5 ± 1 and 6 ± 2%, respectively (Fig. 3, E and F). These data also suggest a net loss of phosphorylated α-syn during its dissociation from the synaptic membrane.
The experiments described above required the co-incubation of membrane and cytosol to assess the transfer of \( \text{Ser(P)-129} \) between fractions. To test whether the loss of the \( \text{Ser(P)-129} \) signal is due to degradation or dephosphorylation during incubation, we first compared the effects of incubating membrane and cytosol separately. These control experiments demonstrated that there was no loss of either cytosolic or membrane-bound \( \text{Ser(P)-129} \) during incubation. Following a 10-min incubation at 37 °C, the level of membrane and cytosolic \( \text{Ser(P)-129} \) was 123% and 122% of starting material, respectively (\( p < 0.05; n = 6 \)) (supplemental Fig. 1A). Thus, loss of the \( \text{Ser(P)-129} \) epitope occurred only during co-incubation of membrane and cytosol, suggesting either that components from both fractions are required for the loss of the \( \text{Ser(P)-129} \) epitope or that dephosphorylation occurred during the transfer of \( \text{Ser(P)-129} \) from one fraction to another. We also assessed whether the loss of \( \text{Ser(P)-129} \) could be mitigated by blockade of endogenous phosphatases in the binding reaction mixture. As shown in supplemental Fig. 1B, a serine/threonine phosphatase inhibitor mixture did not affect the recovery of \( \text{Ser(P)-129} \) on membranes or in cytosol. Therefore, we cannot rule out that some of the \( \text{Ser(P)-129} \) may be preferentially degraded when membrane and cytosol fractions are combined. Alternatively, it remains possible that the phosphatase involved may be insensitive to the inhibitor mixture.

FIGURE 3. \( \text{Ser(P)-129} \) \( \alpha \)-syn dissociation from synaptic membranes. Synaptic membranes isolated from the brains of transgenic mice expressing the human form of WT, A30P, or A53T \( \alpha \)-syn were incubated with \( \alpha \)-syn \( \text{−}^{−} \) cytosol for 10 min at 37 °C. The percentage of membrane-bound and cytosolic \( \alpha \)-syn (WT, A30P, and A53T, A–C, respectively) and Ser(P)-129 \( \alpha \)-syn (WT, A30P, and A53T, D–F, respectively) relative to starting material are displayed.

Does Enzymatic Dephosphorylation or Mutation of Ser-129 Affect \( \alpha \)-syn Interaction with Synaptic Membranes?—The results above suggest that although \( \text{Ser(P)-129} \) levels can be rapidly modulated, phosphorylation does not directly control \( \alpha \)-syn membrane interaction because \( \text{Ser(P)-129} \) loss occurred during both the binding and dissociation events and \( \text{Ser(P)-129} \) was not preferentially associated with either the membrane or cytosolic compartments. Therefore, we asked whether a change in phosphorylation state may be required prior to binding to or dissociation from synaptic membranes.

We first measured the effects of enzymatic dephosphorylation of \( \alpha \)-syn prior to binding to synaptic membranes. We preincubated cytosol prepared from mice overexpressing WT, A30P, or A53T \( \alpha \)-syn with calf intestinal phosphatase (CIP) to dephosphorylate proteins. We then incubated this untreated or CIP-treated cytosol with \( \text{H}^{251} \) \( \alpha \)-syn-deficient membranes and compared the binding capacity of the phosphorylated and dephosphorylated \( \alpha \)-syn. As we reported previously (11), WT and A53T \( \alpha \)-syn showed equivalent binding to synaptic membranes, and both were higher than A30P \( \alpha \)-syn. However, no significant difference in binding capacity was evident between each phosphorylated versus the dephosphorylated \( \alpha \)-syn (Fig. 4, A and B, \( p > 0.05 \), two-way ANOVA).

Second, we assessed whether membrane distribution of \( \alpha \)-syn mutants that mimic dephosphorylated (S129A) or phos-
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Does Manipulation of Phosphorylation at Ser-129 Affect the Membrane Binding of Endogenous α-syn?—As an alternative approach, we manipulated the Ser-129 phosphorylation state of α-syn acutely or chronically in NPC isolated from mice expressing WT α-syn using inhibitors of either PLK2 or the proteasome or by overexpression of PLK2.

Treatment with the PLK2 inhibitor BI2536 (1.0 μM) significantly reduced the level of Ser(129) α-syn relative to GAPDH ~6-fold from 7.3 ± 2.5 to 1.2 ± 0.7% (p < 0.05, one-way ANOVA, see Fig. 5A), but it had no effect on the total amount of membrane-bound α-syn (Fig. 5B). Conversely, exposure to the proteasome inhibitor epoxomicin (20 nM) significantly increased the level of Ser(129) α-syn ~50-fold from 1.3 ± 0.6 to 65 ± 18% (p < 0.05 one-way ANOVA, see Fig. 6A) but had no effect on the levels of membrane-bound α-syn (Fig. 6B). In addition, the epoxomicin-induced increase in Ser(129) α-syn was completely blocked by BI2536, independently of the increased protein ubiquitination and with no effect on membrane binding of α-syn (Fig. 6, A–C), indicating that the accumulation of Ser(129) α-syn was dependent of PLK2-mediated phosphorylation. Following treatment with epoxomicin, Ser(P)-129 levels were elevated such that we could readily detect membrane-bound levels of Ser(P)-129. Of the total Ser(P)-129 α-syn, ~40% was associated with the membranes (data not shown).

To study the long term effects of alterations in levels of Ser(P)-129 α-syn on membrane binding of α-syn, we generated NPC stably expressing His6-tagged human PLK2, which phosphorylates α-syn only at Ser-129 (26), either as WT or a kinase-dead version bearing a K111M mutation. Expression of the epitope-tagged protein was verified WT and K111M cells by both immunofluorescence (Fig. 7A) and Western blotting (Fig. 7C). To confirm the kinase activity of the exogenous PLK2 expression, Ser(P)-129 α-syn was assessed by immunofluorescence in control, WT, and K111M cells (Fig. 7A) and Western blotting (Fig. 7, B and C). Compared with the empty vector controls, Ser(P)-129 α-syn was elevated by WT PLK2 and reduced by K111M PLK2 (p < 0.01 and p < 0.05, respectively, one-way ANOVA). Nevertheless, despite the changes in Ser(P)-129, the proportion of membrane-bound α-syn was similar for the empty vector control (48 ± 1.4%), WT PLK2 (43 ± 3.4%), and K111M PLK2 (42 ± 2.7%) (p < 0.05, one-way ANOVA) (Fig. 7D). Thus, both stable increases and reductions in the extent of α-syn Ser-129 phosphorylation had no effect on the membrane binding capacity of α-syn.

Differential Effect of Ser-129 Phosphorylation on the Membrane Binding of PD-linked α-syn Mutants—Our data above indicate that manipulating Ser-129 phosphorylation has little effect on the binding properties of WT α-syn, suggesting that Ser(P)-129 is likely not a critical, though short lived, modification during membrane binding or dissociation. In previous studies, we showed that both A30P and A53T α-syn have faster membrane dissociation kinetics than WT α-syn and that both mutants also display more transient association upon binding to synaptic membranes (11, 30). Therefore, we asked whether...
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Ser(P)-129 might have any differential effects on the membrane-binding properties of either A30P or A53T α-syn. Because of the relatively low endogenous levels of Ser(P)-129 α-syn, we used epoxomicin to increase acutely the levels of the phosphoprotein. Treatment of NPC expressing WT, A30P, or A53T α-syn with 20 nM epoxomicin for 24 h elevated total Ser(P)-129 α-syn levels but had no effect on total α-syn expression (data not shown). Surprisingly, there was a striking difference in the distribution of WT and mutant phosphopeptides (Fig. 8A). Although the proportion of Ser(P)-129 α-syn that was membrane-bound in WT α-syn cells was 38 ± 1.6%, it was significantly higher in A30P and A53T α-syn cells (79 ± 5.6 and 81 ± 2.3%, respectively) (both p < 0.001, one-way ANOVA) (Fig. 8B). These results suggest that increased Ser-129 phosphorylation may have differential effects on α-syn depending on the presence of PD-linked mutations.

DISCUSSION

Phosphorylation of α-syn at Ser-129 has been observed in post-mortem brain tissue from patients with PD, multiple system atrophy, and neurodegeneration with brain iron accumulation type 1 (15). Under normal physiological conditions, ~4% of α-syn is phosphorylated at Ser-129, whereas ~89% of α-syn is phosphorylated at this residue in LB (15, 40). However, the effects of phosphorylation on the physiological and pathogenic properties of α-syn in vivo remain unknown. In this study, we used two cell-free assays to measure changes to α-syn phosphorylation during binding to or dissociation from synaptic membranes (11, 30). We also analyzed the effects of manipulating Ser(P)-129 α-syn levels by enzymatic, pharmacological, or genetic approaches to assess the impact of Ser-129 phosphorylation on α-syn membrane binding. Our data suggest that the phosphorylation state of α-syn at Ser-129 is highly dynamic, but does not appear to influence the binding of WT α-syn to membranes. We also compared the effects of modifying Ser(P)-129 levels in neural cells expressing PD-linked α-syn bearing A30P or A53T mutations. When Ser-129 phosphorylation in these cells was increased by treatment with the proteasome inhibitor epoxomicin, the phosphorylated α-syn PD mutants showed an increased association with membrane fractions as compared with WT α-syn, suggesting the possibility that the Ser(P)-129 modification may differentially affect α-syn conformation depending on mutations in the amino-terminal membrane binding domain.

We initially investigated the distribution of α-syn and Ser(P)-129 α-syn in brain synaptosomes prepared from transgenic mice expressing human WT, A30P, or A53T α-syn but not the endogenous murine α-syn. Consistent with our previous findings (30), we found that the relative amount of synaptosomal α-syn in the membrane-bound and cytosolic fractions was similar for WT, A30P and A53T α-syn. Experiments in yeast have suggested that A30P α-syn has a lower membrane binding capacity than WT and A53T α-syn (41, 42). However, we have previously shown that cytosolic factors expressed in brain, which yeast may lack, influence the membrane binding ability of recombinant α-syn and can indeed increase the binding capacity of A30P α-syn (11). We also demonstrate that in synaptosomes, there was a lower amount of A30P Ser(P)-129 α-syn, as compared with WT and A53T, in both the membrane-bound and cytosolic fractions. Indeed, these data support those of Zabrocki et al. (29) who also suggest that in yeast reduced Ser(P)-129 A30P α-syn correlates with reduced membrane binding.

As the binding of α-syn to membranes has been shown to be dynamic (30, 43), we investigated changes in the phosphorylation of α-syn at serine 129 during exchange between the membrane and cytosolic compartments. We found that following binding to synaptosomal membranes, there was a loss of Ser(P)-129 α-syn, for WT, A30P, and A53T. A relative loss of Ser(P)-129 α-syn was also observed during dissociation from synaptosomal membranes, suggesting that degradation or dephosphorylation of α-syn may be involved in the binding and dissociation of α-syn. Separate incubations of synaptic membranes or cytosol did not induce a loss of the Ser(P)-129 epitope, arguing that the loss of the phospho-epitope requires both fractions to mix to initiate degradation or dephosphorylation. To shed some light on a role for potential phosphatases, we also included a serine/threonine phosphatase inhibitor mixture in
the binding and dissociation assays. The phosphatase inhibitor mixture failed to block the loss of the Ser(P)-129 signal, raising the possibility that Ser(P)-129 may undergo preferential degradation or that the phosphatase responsible is incompletely inactivated by the inhibitors.

We examined the ability of enzymatically dephosphorylated α-syn to interact with synaptic membranes. There was no significant difference in the membrane binding capacity of α-syn from control cytosol or that containing no Ser(P)-129 α-syn following exposure to CIP, suggesting that phosphorylation at Ser-129 does not directly affect the ability of α-syn to interact with synaptic membranes. This is further supported by our results that there is no difference in the membrane binding capacity of WT α-syn or two Ser-129 mutants of α-syn, one with Ser-129 replaced with alanine (S129A), to prevent phosphorylation, and one with Ser-129 replaced with aspartate (S129D), to mimic the negative electrostatic charge of Ser(P)-129. It has previously been shown that S129A α-syn increases aggregate formation in Drosophila (14). However, more recent studies comparing the two Ser-129 mutants by adenoviral expression in rat brain had differing results. One reported that both mutants had similar aggregation profiles and toxicity (19), and the other suggested that S129A α-syn induced more degeneration than the S129D α-syn, but with no significant differences in the morphology of α-syn aggregates between WT and S129A/D mutants (20). These latter findings are consistent with our results that the membrane binding of S129A and S129D α-syn is similar, although controversy remains regarding the mechanism of toxicity of the S129A and S129D α-syn mutants.

Interestingly, we were unable to find evidence of expression of PLK2 in synaptosomal preparations nor indeed was there any effect of the polo-like kinase inhibitor BI2536 (44) on Ser(P)-129 α-syn levels in synaptosomes (data not shown). We hypothesized that the proteins present in synaptosomes may not represent the full complement of proteins required for modeling α-syn phosphorylation and binding dynamics. Therefore, we made use of NPC isolated from the brains of mice expressing WT α-syn to investigate the effects of manipulating levels of Ser(P)-129 α-syn on membrane binding. Consistent with previous reports (25, 28), 1.0 μM BI2536 inhibited levels of Ser(P)-129 α-syn by ~87% in cultured cells. This significant inhibition of Ser(P)-129 α-syn levels over a 24-h period demonstrates that the phosphorylation of α-syn at Ser-129 is an ongoing process. However, we demonstrate that the membrane-bound α-syn pool in NPC was insensitive to the loss of Ser-129 phosphorylation by BI2536. We further investigated the role of α-syn phosphorylation at Ser-129 in membrane binding by treating NPC with a proteasome inhibitor that has been reported to increase levels of Ser(P)-129 α-syn via stabilization of PLK2 levels (28). Significant increases in Ser(P)-129 α-syn levels by this method also had no effect on membrane binding over a
24-h period. Moreover, additional studies using NPC with stable expression of either WT PLK2 or a K111M mutant with reduced kinase activity produced cell lines with significantly increased or reduced Ser(P)-129 levels, albeit without effects on /H9251-syn membrane binding. Thus, it seems that the membrane binding capacity of /H9251-syn is independent of either acute or chronic increases or decreases in Ser-129 phosphorylation.

Although the early structures of inclusion formation are poorly understood and there remains controversy as to whether /α-syn is phosphorylated before or after aggregation (27, 28), it is notable that the majority of aggregated /α-syn in LB is phosphorylated at Ser-129. Therefore, we also examined the membrane binding capacity of Ser(P)-129 /α-syn itself. Because existing levels of Ser(P)-129 /α-syn were very low in NPC, we took advantage of the fact that a short term exposure to proteasome inhibitors induces a substantial increase in Ser(P)-129 modification as shown in Fig. 6. Following epoxomicin treatment of NPC expressing WT, A30P, or A53T /α-syn, we were able to more clearly compare the membrane binding of WT, A30P, and A53T Ser(P)-129 /α-syn. Interestingly, membrane binding of phosphorylated A30P and A53T /α-syn was significantly higher than for WT Ser(P)-129 /α-syn. It is known that both A30P and A53T mutant forms of /α-syn have a higher propensity to aggregate (45, 46), and indeed these mutants have been known to be phosphorylated at Ser-129 in Lewy bodies of familial forms of PD (18). Our finding that both A53T and A30P /α-syn preferentially accumulate on membranes may underlie the enhanced propensity of these familial PD-related mutants to aggregate.

As phosphorylation of WT /α-syn at Ser-129 appears not to mediate its membrane binding or toxicity, per se, the following
Ser-129 Phosphorylation and Membrane Binding of α-Synuclein

![Ser-129 Phosphorylation and Membrane Binding of α-Synuclein](image)

**FIGURE 8.** Membrane-binding properties of Ser(P)-129 α-syn in WT, A30P, and A53T NPC. NPC (WT, A30P, and A53T) were treated with epoxomicin (Epox) (20 nM) for 24 h. A, representative Western blots showing the level of membrane-bound (M) and cytosolic (C) WT, A30P, and A53T α-syn, and the corresponding Ser(P)-129 α-syn and syntaxin following the epoxomicin treatment are shown. B, level of membrane-bound Ser(P)-129 was quantified by Western blot and shown as a percentage of total α-syn. (*, p < 0.05; ***, p < 0.001 cf. control.)

questions arise. Why does this residue undergo phosphorylation and dephosphorylation and why is the majority of α-syn in LB phosphorylated at Ser-129? Given that Ser-129 is quite distal to the amino-terminal membrane binding region, one possible role for modification of this site may be to regulate interactions with binding partners on synaptic vesicles. For example, the site of binding of α-syn to vesicular synaptobrevin-2 overlaps with the Ser-129 residue (47). Modulation of protein–protein interactions by Ser-129 phosphorylation could thereby impact vesicle mobilization without a direct effect on α-syn membrane binding. With respect to pathological Ser(P)-129 α-syn accumulation, one hypothesis is that α-syn may be phosphorylated after aggregation as it has a higher affinity for kinases and a lower affinity for phosphatases in this altered conformation (28). Furthermore, studies in transgenic mice and cultured cells suggest that cellular toxicity, including pro teaseosomal dysfunction, increases both casein kinase 2 activity and PLK2 leading to enhanced phosphorylation of α-syn at Ser-129 (23, 25).

In conclusion, our data demonstrate that phosphorylation of α-syn at Ser-129 is a continuous cellular process. Although blocking α-syn phosphorylation at Ser-129 or enhancing Ser(P)-129 levels, by enzymatic, pharmacological, and molecular biological means, had little effect on the membrane binding of WT α-syn under either acute or chronic conditions, we obtain contrasting results with the pathogenic α-syn mutants, indicating that membrane association of A30P and A53T α-syn was differentially up-regulated by Ser-129 phosphorylation. This work raises the possibility that the relationship of Ser-129 phosphorylation to the pathogenic aggregation of WT and mutant α-syn may be governed by distinct effects on phospho-protein accumulation on intracellular membranes.

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