Prolyl-isomerase Pin1 Accumulates in Lewy Bodies of Parkinson Disease and Facilitates Formation of α-Synuclein Inclusions**

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Parkinson disease (PD)2 is a relatively common neurodegenerative disorder that is characterized by the loss of dopaminergic neurons and by the formation of Lewy bodies (LBs), which are cytoplasmic inclusions containing aggregates of α-synuclein. Although certain post-translational modifications of α-synuclein and its related proteins are implicated in the genesis of LBs, the specific molecular mechanisms that both regulate these processes and initiate subsequent inclusion body formation are not yet well understood. We demonstrate in our current study, however, that the prolyl-isomerase Pin1 localizes to the LBs in PD brain tissue and thereby enhances the formation of α-synuclein immunoreactive inclusions. Immunohistochemical analysis of brain tissue from PD patients revealed that Pin1 localizes to 50–60% of the LBs that show an intense halo pattern resembling that of α-synuclein.

Utilizing a cellular model of α-synuclein aggregation, we also demonstrate that, whereas Pin1 overexpression facilitates the formation of α-synuclein inclusions, dominant-negative Pin1 expression significantly suppresses this process. Consistent with these observations, Pin1 overexpression enhances the protein half-life and insolubility of α-synuclein. Finally, we show that Pin1 binds synphilin-1, an α-synuclein partner, via its Ser-211-Pro and Ser-215-Pro motifs, and enhances its interaction with α-synuclein, thus likely facilitating the formation of α-synuclein inclusions. These results indicate that Pin1-mediated prolyl-isomerization plays a pivotal role in a post-translational modification pathway for α-synuclein aggregation and in the resultant Lewy body formations in PD.

Parkinson disease (PD)2 is one of the most common neurodegenerative disorders and is characterized by the loss of dopaminergic neurons in the substantia nigra and by the presence of cytoplasmic inclusions known as Lewy bodies (LBs) in surviving neurons (1, 2). LBs have classically been considered as a pathological hallmark of PD, consisting of many components, including α-synuclein, which is one of the major constituents (3, 4). The first indication of a pathogenic role for α-synuclein in PD came from the results of linkage analysis of mutations in its gene in autosomal dominant forms of the disease (5, 6). α-Synuclein is an unfolded protein in its native state, but in a pathological state it can be induced to form either α-helical or β-sheet structures that result in the formation of insoluble α-synuclein aggregates (7, 8). The aggregation of α-synuclein can be modified by a range of factors, both in vitro and in vivo, including environmental regulators of pH, temperature, ionic strength, and oxidative stress and by intrinsic intracellular pathways (8). The latter of these regulatory networks includes several α-synuclein-binding proteins such as synphilin-1 (9) and post-translational modifications of related molecules, such as phosphorylation and ubiquitination (10–12). Synphilin-1 was identified as a protein that interacts with α-synuclein and has been shown to be a substrate of the PD-associated ubiquitin ligases, Parkin, SIAH, and dorfin (9, 13–16). Recent studies indicate that synphilin-1 ubiquitination via these ubiquitin ligases is important for the formation of LBs (13, 14, 17). In fact, the co-expression of the α-synuclein and synphilin-1 proteins results in the formation of cytoplasmic inclusions in a subset of cultured cells (9, 18). However, it is currently not well understood how these two molecules function cooperatively during inclusion body formation or how their functional interaction is regulated.

Phosphorylation-dependent prolyl-isomerization is a recently characterized post-translational modification mechanism that regulates the function and properties of specific proteins (19, 20). These modifications are catalyzed by a peptidyl prolyl-isomerase, Pin1, which specifically binds phosphorylated serine or threonine residues immediately preceding proline (pSer/Thr-Pro motifs) in a subset of proteins, and promotes the cis/trans isomerization of the peptide bond (19, 20). Such conformational changes have been shown to have profound effects on the function of Pin1 substrates as they can modulate catalytic activity, phosphorylation status, protein-protein interactions, subcellular localization, and protein stability (21, 22). Consequently, Pin1 has been shown to be involved in the regulation of many cellular events, including proliferation, differentiation, and cell death, and has been reported to be associated with several human diseases, including cancer and Alzheimer disease (AD) (19, 23, 24). In AD brain tissue, Pin1 accumulates in pathological neurofibrillary tangles, thereby specifically interacting with phosphorylated tau proteins on their Thr-231-Pro motifs (24–26). Sig-
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significantly, by catalyzing the cis/trans-isomerization of tau, Pin1 restores the ability of phosphorylated tau to bind microtubules and promotes tau dephosphorylation by the PP2A phosphatase (25, 26). However, although the crucial role of Pin1 in the pathogenesis of AD has begun to be characterized, its involvement in other neurodegenerative diseases is unknown.

In our current study, we provide evidence that Pin1 plays a role in the aggregation and degradation of α-synuclein. We show that Pin1 accumulates in the LBs of PD tissue and co-localizes with α-synuclein in a cell culture model of α-synuclein inclusions. Furthermore, the overexpression of Pin1 enhances the formation of α-synuclein inclusions, which is accompanied by their increased half-life and insolubility. We also demonstrate that Pin1 interacts with synphilin-1 in a phosphorylation-dependent manner and regulates its interaction with α-synuclein. These results together indicate that Pin1-mediated prolyl-isomerization may be involved in the formation of LBs and, consequently, in the pathogenesis of PD.

EXPERIMENTAL PROCEDURES

Immunochemistry—The sampling and usage of all brain tissues in this study was performed under the guidelines of the committee in Yokohama City University for the use of clinical samples, protocol no. 04-008. A total of six autopsied brains from patients with PD and five normal controls were examined in this study. Tissue sections were placed on slides, deparaffinized in xylene, hydrated in 100 and 75% ethanol, and then washed with PBS. The tissue slides were pretreated with 99% formic acid for 3 min. After extensive washing with PBS, the slides were then treated with PBS containing 5% goat serum and 0.1% Triton X-100 for blocking and then incubated with either anti-Pin1 polyclonal antibodies (1:500) (21) or anti-α-synuclein polyclonal antibodies (1:100) (27) at 4 °C in humidified chamber for 12 h. After washing with PBS, the slides were then incubated with biotinylated secondary antibody for 2 h. Immunohistochemical analysis was then performed using Vectastain ABC kit and DAB-staining solution (Vector Laboratories, Burlingame, CA).

Protein Degradation Assay—293T cells were transfected with α-synuclein, with Xpress-LacZ used as a control. Cycloheximide (100 μg/ml) was added to the media 24 h after transfection, and cells were harvested at different time points. Total cell lysates in SDS sample buffer were sonicated and then analyzed by immunoblotting with an anti-Xpress (Invitrogen) or anti-α-synuclein antibodies (27). The blots were scanned and semiquantified using National Institutes of Health Image 1.6.2 software (22).

GST Pull-down Assay, Immunoprecipitation, and Immunoblotting Analyses—293T and COS-1 cells were lysed with GST pull-down buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 1 mM Na3VO4, 1 mM diithreitol, 0.5 μg/ml leupeptin, 1.0 μg/ml pepstatin, and 0.2 μM phenylmethylsulfonyl fluoride) and incubated with 30 μl of glutathione-agarose beads containing either GST-Pin1 or GST at 4 °C for 2 h. The precipitated proteins were then washed three times with lysis buffer and subjected to SDS-PAGE. For immunoprecipitation, cells were harvested 24 h after transfection and lysed with Nonidet P-40 lysis buffer (10 mM Tris HCl (pH 7.5), 100 mM NaCl, 0.5% Nonidet P-40, 1 mM Na3VO4, 100 mM NaF, 0.5 μg/ml leupeptin, 1.0 μg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated for 1 h with Protein A/G-Sepharose/non-immunized IgG complexes. Supernatant fractions were recovered and immunoprecipitated with 5 μg of anti-HA or anti-Xpress antibody and 30 μl of Protein A/G-Sepharose. After washing three times with lysis buffer, pellets were analyzed on SDS-PAGE gels and subjected to immunoblot analysis. For GST-pull-down assay with in vitro translated proteins, pcDNA-synphilin-1 and pcDNA-synphilin-1(Δ207–216) were translated in vitro with the TNT-coupled transcription/translation kit (Promega). They were then incubated in Xenopus extracts, as described previously (21). Proteins translated in vitro were incubated with 20 μl of glutathione-agarose beads containing GST-Pin1 or GST at 4 °C for 2 h, as described previously (21). The precipitated proteins were washed with GST-lysis buffer and subjected to SDS-PAGE.

Construction of Synphilin-1 and α-Synuclein Vectors—The cloning of full-length synphilin-1 cDNA was performed as described previously (28). Synphilin-1 cDNA was further subcloned into the pcDNA-HisMax vector (Invitrogen) to generate N-terminal Xpress-tagged synphilin-1. α-Synuclein cDNA was amplified by PCR from a human brain cDNA library and inserted into either the pcDNA or pcDNA-HA expression vector (Invitrogen) to generate untagged or C-terminal HA-tagged α-synuclein, respectively.

Immunofluorescent Analysis—Exponentially growing 293T cells on coverslips were transfected with pcDNA-α-synuclein and pcDNA-synphilin-1 using Effectene reagent (Qiagen), according to the manufacturer’s instructions. 24 h after transfection, 10 μl of MG-132 was added to the culture medium for the final 12 h of culturing. Cells were then fixed with 3% formaldehyde and treated with PBS, containing 5% goat serum and 0.1% Triton X-100 for blocking, and then incubated with anti-α-synuclein polyclonal antibodies at room temperature in a humidified chamber for 2 h (27). After washing with PBS, slides were incubated with anti-rabbit-Alexa568 secondary antibody (Molecular Probes) for 1 h followed by immunofluorescent analysis using fluorescent laser microscopy (Olympus, Tokyo, Japan). More than 200 GFP-positive cells per slide were scored for inclusion body formation.

Phospho-peptides Pull-down Assay—1 μg of Biotin-labeled phosphorylated peptides was captured with 30 μl of streptavidin magnetic beads (New England Biolabs) and then mixed with 2 μg of recombinant GST or GST-Pin1 in GST-lysis buffer. After a 1-h incubation at 4 °C, the beads were washed three times with GST-lysis buffer and then incubated with 2× sample buffer with boiling for 5 min. The supernatant fractions were then subjected to immunoblotting analysis with anti-Pin1 antibody.

Construction of Synphilin-1 siRNA—Synphilin-1 siRNAs were created as described previously (29) with the following sequences: synphRNAiA (GATTCGGAATGTCGCTTTAT); synphRNAiB (CTCACCTCCTCTGGTTAAAA); and control-RNAi (TCGTATGTGTGT-GGAATT).

RESULTS

Pin1 Accumulates in the Lewy Bodies of Parkinson Disease—The pathogenesis of PD has been implicated in the formation of LBs, accompanied by specific post-translational modifications of related molecules (2, 10). Because the prolyl-isomerase Pin1 regulates a broad range of post-translational modification processes (19, 23), we hypothesized that it may play some role also in the pathogenesis of PD. To test this hypothesis, we first attempted immunohistochemical analyses of PD brain tissue from affected patients with both anti-Pin1 and anti-α-synuclein antibodies. We subsequently found that the anti-Pin1 antibody positively stained the LBs in the midbrains of each of the PD patients and that 50–60% of these LBs were detectable by this immunostaining (Fig. 1A). In addition, Pin1 was observed to be localized more intensely in the halo of the majority of the LBs, whereas the neuronal cytoplasm was very weakly stained (Fig. 1, B and C). Anti-α-synuclein antibody
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Pin1 Binds and Co-localizes with α-Synuclein in Intracellular Inclusions—Our observations, that Pin1 localizes to LBs and that its staining pattern resembles that of α-synuclein, suggested the possibility that these two proteins interact in these structures. We therefore examined the interaction between Pin1 and α-synuclein in cultured cells. 293T cells were transfected with HA-tagged α-synuclein, and lysates from these cells were immunoprecipitated with an anti-HA antibody. The resulting immunoblotting analysis revealed that endogenous Pin1 does indeed bind to α-synuclein (Fig. 2A). To further confirm this specific interaction in vitro, GST-pull-down experiments were performed. Lysates of 293T cells that had been transfected with α-synuclein were incubated with either GST or GST-Pin1. α-Synuclein was subsequently detected in GST-Pin1-bound agarose beads, but not the control GST beads (Fig. 2B). This further indicated that a specific interaction occurs between Pin1 and α-synuclein.

Pin1 has been shown to specifically bind motifs containing a phosphorylated serine or threonine preceding proline (Ser/Thr-Pro). Because α-synuclein has no such motif, we speculated that Pin1 binding to α-synuclein could be indirect. To address this question, we performed an in vitro binding assay. Recombinant His-tagged α-synuclein or tau proteins were treated with Xenopus mitotic extracts and then subjected to GST-pull-down analysis as performed previously (21). As shown in Fig. 2C, although both proteins were well phosphorylated with Xenopus extracts, only tau, but not α-synuclein was pulled down with GST-Pin1. These data therefore show that Pin1 associates with α-synuclein in cell lysates but that it cannot bind to α-synuclein in experiments using purified recombinant proteins. This suggests that there is an indirect interaction between these two proteins.

It has been previously shown that the co-transfection of α-synuclein and synphilin-1 results in the formation of intracellular inclusions in cultured cells (9, 14). We utilized this finding to investigate whether Pin1 co-localizes with α-synuclein in these inclusions. 293T cells were co-transfected with α-synuclein, synphilin-1, and GFP-Pin1, followed by immunocytochemical analysis. Pin1 was again found to co-localize with α-synuclein in intracellular inclusions that showed either halo or spot patterns (Fig. 2, D and E), as described previously (9, 14). However, Pin1 overexpression alone did not lead to the formation of either aggregates or inclusions with other proteins (supplemental Fig. S1). These results further indicate that Pin1 indirectly interacts with α-synuclein in intracellular inclusions.

Pin1 Facilitates the Formation of α-Synuclein Immunoreactive Inclusions—We next addressed whether Pin1 overexpression affects the formation of α-synuclein inclusions. It has been shown in earlier studies that the co-expression of α-synuclein and its binding partner synphilin-1 in mammalian cells results in the formation of cytoplasmic inclusions composed of α-synuclein (9, 14). We expanded upon this finding to investigate whether Pin1 has any effects on inclusion body formation. 293T cells were transfected with α-synuclein and synphilin-1, together with either GFP, GFP-Pin1, or a GFP-dominant negative (dn)-Pin1, and examined for α-synuclein immunopositive inclusions. The co-expression of GFP-Pin1 in this experiment resulted in a significant increase in the formation of α-synuclein immunoreactive inclusions and in the numbers of inclusion positive cells, when compared with control GFP-transfected cells (Fig. 3, A and C). In contrast, when a dominant-negative Pin1 construct was co-expressed in parallel experiments, there were significant decreases in the number of cells containing α-synuclein inclusions (Fig. 3, A and C). These phenomena were also confirmed by an additional experiment with non-tagged Pin1 constructs (data not shown). However, transfection of GFP, GFP-Pin1, or GFP-dnPin1 without co-transfection of α-synuclein and synphilin-1 did not result in the inclusion formations described by previous studies (supplemental Fig. S1). Likewise, hematoxylin and eosin staining also revealed in parallel experiments that GFP-Pin1 enhances, whereas GFP-dnPin1 decreases, the number of cells with eosinophilic cytosolic inclusions (Fig. 3B). These results suggested that a specific functional interaction exists between Pin1 and α-synuclein in the formation of α-synuclein inclusions. To address whether either the binding or the catalytic activity of Pin1 is required for inclusion body formation, we performed an experiment using either a WW-domain (binding domain) mutant (W34A) or peptidyl-prolyl-cis/trans-isomerase domain (catalytic domain) mutant (K63A) of Pin1. The subsequent inclusion body formation analysis revealed that both of these Pin1 mutants failed to enhance the formation of α-synuclein inclusions (Fig. 3D), indicating that both domains are required for inclusion body formation. Taken together, our results indicate that Pin1 plays a role in the formation of intracellular α-synuclein inclusions.

Pin1 Enhances the Protein Half-life and Insolubility of α-Synuclein—Our data showing that Pin1 interacts with α-synuclein and promotes α-synuclein inclusion formation prompted us to examine the
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FIGURE 2. Pin1 indirectly interacts and co-localizes with α-synuclein in inclusion bodies. A, 293T cells were transiently transfected with HA-α-synuclein and Pin1. Lysates were subjected to immunoprecipitation with the anti-HA 12CA5 antibody, followed by either anti-Pin1 or anti-α-synuclein immunoblotting (lower panel). B, 293T cells expressing pcDNA-α-synuclein were subjected to GST-pull-down assays with either GST or GST-Pin1, followed by anti-α-synuclein immunoblotting. C, Pin1 cannot bind directly to α-synuclein. Recombinant His-tagged α-synuclein or tau protein were treated with mitotic Xe after 36 h. Pin1 (green fluorescence) and α-synuclein (red fluorescence) co-localize in inclusions (merged image) showing halo (D) or spot patterns (E). "Triple" indicates a merged image with 4′,6-diamidino-2-phenylindole staining.

Possible function of Pin1 in the post-translational modification of the α-synuclein protein. We initially examined both the stability and the solubility of α-synuclein, because these two properties are important for aggregate formation. 293T cells were transfected with α-synuclein, together with either a GFP vector control or GFP-Pin1 construct, followed by treatment with cycloheximide to inhibit protein synthesis. We then harvested the cells at different time points and examined α-synuclein protein levels by Western blot analysis. Interestingly, Pin1 overexpression significantly inhibited the degradation of α-synuclein (Fig. 4A). We next examined whether Pin1 affects the detergent solubility of α-synuclein and performed parallel detergent fractionation analysis as reported previously (30). We found that Pin1 overexpression increases the accumulation of Triton X-insoluble α-synuclein and decreases the Triton X-100-soluble fraction (Fig. 4B). This is consistent with the finding of increased inclusion formation by Pin1. Taken together, these results indicate that Pin1 enhances the protein half-life and insolubility of α-synuclein, both of which could be predicted to contribute to the formation of α-synuclein inclusions.

Pin1 Binds Phosphorylated Synphilin-1 on Ser-211-Pro and Ser-215-Pro—To investigate the molecular mechanism by which Pin1 affects the formation of α-synuclein inclusions, we examined whether Pin1 associates with synphilin-1, because this protein is a major binding partner of α-synuclein and contributes to the generation of α-synuclein inclusions (9, 13, 14). Significantly, both immunoprecipitation and GST-pull-down analyses revealed that Pin1 can specifically interact with synphilin-1 (Fig. 5, A and B). Moreover, this binding was abolished by pretreatment of the cell lysates with A-phosphatase (Fig. 5B), indicating that Pin1 interacts with phosphorylated synphilin-1. Furthermore, a WW-domain mutant of Pin1 (W34A) failed to bind synphilin-1, indicating the requirement of this domain for this interaction (Fig. 5C).

To elucidate the Pin1-binding site(s) in synphilin-1, we constructed and then expressed four different synphilin-1 deletion mutants. When these mutants were expressed in COS-1 cells followed by a GST-pull-down assay, only mutants A and D, but not mutants B or C, bound GST-Pin1 (Fig. 5D). This indicated a specific interaction of Pin1 with the N-terminal region of synphilin-1. Because the mutant A fragment contains five putative Pin1 binding motifs, we created five biotinylated phospho-peptides each containing one of these phospho-Ser-Thr-Pro motifs (Fig. 5E). When these peptides were fixed on streptavidin beads and incubated with GST-Pin1, only peptides including Ser-211-Pro and Ser-215-Pro were found to capture GST-Pin1 (Fig. 5E and data not shown). We next created a minimal synphilin-1 deletion mutant (Δ207–216) devoid of both of these Pin1 binding sites. When this mutant was synthesized in vitro and phosphorylated by Xenopus extracts, followed by a GST-pull-down assay, this abrogated the binding to Pin1, although wild-type synphilin-1 was still able to bind (Fig. 5F). These data show that Pin1 binds phosphorylated synphilin-1 on its Ser-211-Pro and Ser-215-Pro motifs. We next addressed whether this synphilin-1 mutant that is defective in Pin1 binding sites is in fact unresponsive to Pin1. 293T cells were transfected with α-synuclein and either wild-type synphilin-1 or its Δ207–216 mutant and co-transfected with either GFP or GFP-Pin1. Indirect immunofluorescence analyses subsequently revealed that, although the Δ207–216 mutant could still function in the formation of α-synuclein inclusions at a basal level, this mutant was no longer responsive to Pin1 in this process (Fig. 5G). This suggests a functional interaction between Pin1 and synphilin-1 in α-synuclein inclusion formation.

We next tested the effects of several different Ser/Thr kinase inhibitors on Pin1 binding to synphilin-1. COS-1 cells were transiently transfected with Xpress-synphilin-1 followed by treatment either with the casein kinase (CK) II inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB), the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor U0126, the cyclin-dependent kinase inhibitor roscovitine, or a Me2SO control for 12 h. Lysates were then subjected to immunoprecipitation with anti-Xpress antibody followed by immunoblot analysis with anti-Pin1 antibody. We observed that only DRB treatment resulted in a marked reduction of the binding between Pin1 and synphilin-1 (Fig. 5F). This suggested that CKII-mediated phosphorylation is important for this interaction and may be required to generate the correct binding module for Pin1.
We next tested whether Pin1 binding to α-synuclein might occur via synphilin-1. A two-step immunoprecipitation experiment revealed that Pin1 associates with α-H9251-synuclein bound to synphilin-1 but not with free α-H9251-synuclein (Fig. 5I), indicating that this association occurs via synphilin-1. Furthermore, targeted depletion of endogenous synphilin-1 by two different siRNAs abolished the interaction of α-H9251-synuclein with Pin1 in an immunoprecipitation assay (Fig. 5J), again suggesting that Pin1 associates with α-H9251-synuclein via synphilin-1.

Pin1 Promotes the Interaction of α-H9251-Synuclein with Synphilin-1—We next addressed whether Pin1 expression affects the interaction between α-synuclein and synphilin-1, because this binding is crucial for the formation of cytoplasmic inclusions (9, 31). 293T cells were transfected with HA-α-synuclein and Xpress-synphilin-1, in the presence or absence of exogenous Pin1, and lysates were subjected to immunoprecipitation with anti-Xpress antibodies. In cells overexpressing Pin1, significantly more α-H9251-synuclein was detected in the anti-Xpress immunoprecipitates (Fig. 6A). To further confirm these results, we examined the effects of Pin1 on α-synuclein and synphilin-1 binding in vitro. 293T cells were transfected with Xpress-synphilin-1 and either Pin1 or a vector control and then subjected to GST-pull-down analysis with a recombinant GST-α-synuclein protein. Pin1 overexpression was found to enhance the binding of synphilin-1 with GST-α-synuclein (Fig. 6B). These results together indicate that Pin1 positively regulates the interaction between α-synuclein and synphilin-1, thereby likely resulting in the enhanced formation of α-synuclein inclusion bodies.

DISCUSSION

In the current study, we demonstrate that: 1) Pin1 is a component of 50–60% of the Lewy bodies that are a hallmark of Parkinson disease; 2) Pin1 indirectly interacts and co-localizes with α-synuclein in inclusions in cells; 3) Pin1 promotes, whereas dominant-negative Pin1 abrogates, the formation of α-synuclein inclusions; 4) Pin1 inhibits the degradation of α-synuclein, which is accompanied by an increase in Triton-X insoluble α-synuclein; 5) Pin1 binds phosphorylated synphilin-1 on its Ser211-Pro and Ser215-Pro motifs, which may be affected by CKII; and 6) Pin1 enhances the interaction between α-synuclein and synphilin-1 (Fig. 6C).

Phosphorylation-dependent prolyl-isomerization plays a pivotal role in many cellular regulatory mechanisms (19). Pin1-mediated conformational changes can regulate a series of post-translational modifications of substrate proteins, including phosphorylation (or de-phosphorylation), ubiquitination, protein-protein interactions, subcellular localiza-
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A

GFP

GFP-Pin1

α-synuclein

LacZ

0 4 8 12 16

0 4 8 12 16

Relative protein levels

0 1 2 3 4 5 6 7

Chase time (hours)

FIGURE 4. Pin1 enhances both the protein half-life and insolubility of α-synuclein. A, Pin1 inhibits the degradation of α-synuclein. 293T cells were co-transfected with α-synuclein, Xpress-LacZ and either a pIRE5-GFP vector control or pIRE5-GFP-Pin1 construct. At 24 h after transfection, cells were treated with cycloheximide (100 μg/ml) and harvested at the indicated time points. The cells were then lysed with SDS-Laemmli sample buffer and subjected to anti-α-synuclein or anti-Xpress immunoblotting. B, Pin1 enhances the insolubility of α-synuclein. Cells were co-transfected with synphilin-1 and either a GFP vector control or GFP-Pin1, and then harvested 24 h later. Cells were fractionated into Triton X-100-soluble or insoluble (SDS-lysis buffers soluble) compartments and then subjected to immunoblotting analysis with anti-α-synuclein antibodies.

tion, and protein stability (19, 21, 22). Consequently, Pin1 has been shown to be involved in the regulation of many cellular events, such as proliferation, differentiation, and cell death, and in the genesis of certain diseases such as cancer and neurodegenerative disorders (19, 23). Alzheimer disease (AD) is the best recognized example of a brain disorder in which Pin1 plays a pathological role in the formation of neurofibrillary tangles (24, 25). In AD, hyperphosphorylation of the tau protein results in impaired microtubule assembly and polymerization. Hyperphosphorylated tau proteins are therefore the main components of the aggregated filaments found in neurofibrillary tangles (32). Pin1 binds the phosphorylated Thr-231-Pro motif of the tau protein and restores the function of tau by enhancing its dephosphorylation by phosphatase 2 (PP2) (26). This Pin1-mediated post-translational modification of tau consequently reduces the formation of neurofibrillary tangles (24, 25).

In contrast to AD, we show here that Pin1 enhances the formation of the LB cytoplasmic inclusions in PD. This discrepancy in the function of Pin1 for cytoplasmic inclusions between these two neurodegenerative diseases may be explained by the accumulating evidence that Pin1 affects different substrates in different cellular and physiological and/or pathological contexts, where the phosphorylation patterns of potential Pin1 targets have been altered (20, 25). In the pathological context of PD, the dopaminergic neurons in the substantia nigra or in the locus ceruleus may be affected such that Pin1 has a higher affinity for the α-synuclein-synphilin-1 complex than for tau, such that it does not co-localize with the tau protein in LBs (data not shown).

Multiple α-synuclein-interacting proteins that modulate the formation of α-synuclein inclusions have now been reported. These include histones, tubulin, the brain-specific protein p25, and molecular chaperones such as HSP70 and CHIP (8, 30, 33–36). The identity of the individual molecules that may affect α-synuclein aggregation, the molecular etiology of LB formation, and the resulting impact on neuronal survival are still elusive. However, the evidence that the co-expression of α-synuclein and synphilin-1 leads to LB-like inclusion body formation in cultured cells may suggest that the functional interaction between these factors is crucial for this process (9). Therefore, it is reasonable to predict that the identification of factors that regulate the physical and functional interactions between α-synuclein and synphilin-1 will help to uncover the molecular etiology of LB formation and PD pathogenesis.

CKII has been recently reported as a potent kinase that phosphorylates both α-synuclein and synphilin-1 and regulates the binding between these two proteins (11, 31). Significantly, the CKII inhibitor, DRB, abolishes this interaction and reduces inclusion body formation in cell cultures (31). Consistent with this observation, we show in our current study that Pin1 binds phosphorylated synphilin-1 and that this is abolished by DRB. This indicates that phosphorylation of synphilin-1 or other proteins by casein kinase II may be a prerequisite for both Pin1 binding and catalysis of α-synuclein-synphilin-1 complexes. Furthermore, these results indicate that CKII phosphorylation and subsequent Pin1-mediated prolyl-isomerization of synphilin-1 may cooperatively promote α-synuclein inclusion formation. Interestingly, neither of the Pin1-binding motifs on synphilin-1, Ser-211-Pro, and Ser-215-Pro, seem to be potential CKII phosphorylation sites. Moreover, a previous study showed that Pin1 directly interacts with CKII (37), indicating that a more complex regulatory mechanism may exist for the Pin1 regulation by CKII.

We also demonstrate in this study that Pin1 inhibits the degradation of α-synuclein and that this is accompanied by the accumulation of Triton X-100-insoluble protein. Previous studies demonstrated that chaperones such as Hsp70 prevent the accumulation of Triton X-100-insoluble α-synuclein and enhance its degradation in transgenic mice.
FIGURE 5. Pin1 binds phosphorylated synphilin-1. A, Pin1 interacts with synphilin-1 in vivo. COS-1 cells were co-transfected with Xpress-synphilin-1 and FLAG-Pin1, followed by immunoprecipitation with anti-Xpress antibodies. Immunoprecipitates were then subjected to immunoblotting analysis with either anti-Pin1 or anti-synphilin-1 antibodies.

B, Pin1 binds phosphorylated synphilin-1. COS-1 cells were transfected with synphilin-1 and harvested 24 h after transfection. Cell lysates were then treated with either PBS or H9261 phosphatase and subjected to GST-pull-down analysis with either GST or GST-Pin1, followed by the anti-synphilin-1 immunoblotting. C, COS-1 cells were transfected with synphilin-1, and cell lysates were subjected to GST-pull-down analysis with either GST-Pin1 or GST-Pin1W34A, followed by anti-synphilin-1 immunoblotting. D, Pin1 binds the N-terminal region of synphilin-1. COS-1 cells transfected with full-length synphilin-1, or its truncated mutants as shown in the upper panel, were subjected to a GST-pull-down assay, followed by anti-synphilin-1 immunoblotting. The specific binding of Pin1 to pSer-211-Pro and pSer-215-Pro motifs in synphilin-1. Phosphorylated peptides incorporating the indicated single putative Pin1-binding motif (pSer/Thr-Pro) were captured by streptavidin magnetic beads, followed by co-precipitation with GST-Pin1. Co-precipitates were analyzed by anti-Pin1 immunoblotting, and the results are depicted on the right side of the panel. E, in vitro translated synphilin-1, and its minimal deletion mutant devoid of Pin1-binding sites (synphilin-1Δ207–216), were treated with mitotic Xenopus extracts and subjected to GST-pull-down analysis followed by anti-synphilin-1 immunoblotting. G, 293T cells were co-transfected with α-synuclein and synphilin-1 (both WT and the Δ207–216 mutant), and either GFP or GFP-Pin1 constructs. Immunoblotting analysis was then undertaken with the antibodies listed in the upper panel. The number of cells containing inclusion bodies is expressed relative to the number of transfected cells and normalized to GFP protein levels (lower panel). Error bars represent the standard deviation of three independent experiments. H, Pin1 binding to synphilin-1 is dependent on casein kinase (CK) II. COS-1 cells were transfected with Xpress-synphilin-1 and treated with either DRB (50 μM), roscovitine (25 μM), U0126 (1 μM), or Me2SO vehicle alone. Cell lysates were subjected to immunoprecipitation with anti-Xpress antibody followed by immunoblotting with either anti-Pin1 or anti-synphilin-1 antibodies. I, Pin1 binds α-synuclein bound to synphilin-1. COS-1 cells were co-transfected with HA-α-synuclein, Xpress-synphilin-1, and FLAG-Pin1. Cell lysates were then co-immunoprecipitated with anti-Xpress antibody (1st IP). The supernatant fraction was recovered and then subjected to immunoprecipitation with HA antibody (2nd IP). Immunoprecipitates were analyzed by immunoblotting with either anti-α-synuclein, anti-synphilin, or anti-Pin1 antibodies. J, 293T cells were infected with either a retrovirus containing control siRNA or two different synphilin-1-specific siRNAs (synph-RNAiA or synph-RNAiB), and infectants were selected by puromycin for 48 h. Cells were then transfected with HA-α-synuclein and Pin1 followed by immunoprecipitation with anti-HA antibody. Immunoprecipitates were separated by SDS-PAGE and subjected to immunoblotting analysis with anti-α-synuclein or anti-Pin1 antibodies.

**Pin1 Enhances Formation of α-Synuclein Inclusions**
and in Drosophila (30, 38). Hence, the effects of Pin1 upon α-synuclein solubility may antagonize its protein folding by molecular chaperones. Further detailed structural and biochemical analysis will need to be performed to determine how Pin1 affects α-synuclein solubility and protein half-life and to elucidate the α-synuclein degradation pathway, i.e. the proteasomal or lysosomal pathway in which Pin1 is involved (10, 39).

It is of interest to address the question of why Pin1 is a component of only 50–60% of Lewy bodies (Fig. 1A). We speculate that the antigenicity of the Pin1 protein might be diminished in tandem with the maturation of the Lewy bodies or that Pin1 may be released from LBs during a particular stage of their formation. Because, in our cellular system, Pin1 was found to be localized with almost all of the α-synuclein inclusions (Fig. 3A and data not shown), the former of these possibilities may be the case. Interestingly, a recent report has shown that only 30% of the Lewy bodies from the substantia nigra of PD patients were SIAH-positive, despite the evidence for the significant role of SIAH in inclusion body formation (14). Therefore, the significance of Pin1 function for inclusion formation may not correlate with the ratio of Pin1-positive LBs. Further detailed in vivo studies will be necessary to address this issue by revealing the underlying etiology of LB formation.

Lewy bodies had initially been described as only pathological hallmarks of Parkinson’s disease (40). However recent studies utilizing genetic models and cell culture systems have revealed a role for LBs in both cytotoxicity and neuronal death (2, 7). The question of whether intracellular inclusions protect against, or contribute to these cytotoxic effects is still, however, somewhat controversial (41, 42). Recent analyses with cell cultures have demonstrated that LB-like inclusion bodies are present mainly in surviving cells and very few are detectable in apoptotic cells (41). Furthermore, our unpublished observations and other previous reports indicate that cells containing inclusions have normal morphologies and intact nuclei, with no evidence of apoptosis (31). This suggests that the inclusions themselves may not be cytotoxic. These findings are consistent with experimental evidence from transgenic Drosophila models of the phosphorylated and non-phosphorylated forms of α-synuclein, indicating a lack of correlation between inclusions and neuronal death, but rather a protective effect of the LBs against neuronal death via the sequestration of toxic phosphorylated α-synuclein (42). Hence, it is difficult to conclude whether Pin1 protects or enhances cell death in the dopaminergic neurons of PD brains as a consequence of enhanced LB formation. Further in vivo studies using Pin1 transgenic or knock-out mice with other PD mouse models will be required.

In summary, we demonstrate here that Pin1 accumulates in the Lewy bodies of Parkinson disease and co-localizes with α-synuclein at intracellular inclusions in affected cells. Moreover, Pin1 inhibits the degradation of α-synuclein and facilitates the formation of α-synuclein inclusions. These findings indicate that Pin1 is a major component of LBs and affects the genesis of α-synuclein aggregates, and is thus an important key factor in the pathogenesis of PD.

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