Prolyl oligopeptidase enhances α-synuclein dimerization via direct protein-protein interaction

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ABSTRACT

Prolyl oligopeptidase (PREP) accelerates the aggregation of α-synuclein (aSyn), a key protein involved in development of Parkinson’s disease and other synucleinopathies. PREP inhibitors reduce aSyn aggregation but the mechanism has remained unknown. We have now used protein-fragment complementation assays (PCA) and microscale thermophoresis (MST) in parallel to show that PREP interacts directly with aSyn in both intact cells and in a cell-free system. Using split luciferase-based PCA, we first showed that PREP enhances the formation of soluble aSyn dimers in live N2A neuroblastoma cells. A PREP inhibitor, KYP-2047, reduced aSyn dimerization in PREP-expressing cells but not in cells lacking PREP expression. aSyn dimerization was also enhanced by PREP(S554A), an enzymatically inactive PREP mutant, but this was not affected by KYP-2047. PCA and MST studies showed that aSyn interacts with both PREP and PREP(S554A) with low micromolar affinity. Neither the proline-rich, C-terminal domain of aSyn or the hydrolytic activity of PREP were required for the interaction with PREP. Our results show that PREP binds directly to aSyn to enhance its dimerization, and may thus serve as a nucleation point for aSyn aggregation. Native gel analysis showed that KYP-2047 shifts PREP to a compact monomeric form with reduced ability to promote aSyn nucleation. As PREP inhibition also enhances autophagic clearance of aSyn, PREP inhibitors may reduce accumulation of aSyn inclusions via a dual mechanism, and are thus a novel therapeutic candidate for synucleinopathies. Our results also suggest that PREP has other cellular functions in addition to its peptidase activity.

INTRODUCTION

Accumulation of α-synuclein (aSyn) containing protein inclusions in the brain is the defining feature of synucleinopathies. In the most common neurodegenerative disorder with movement deficits, Parkinson’s disease (PD), and in dementia with Lewy bodies they are called Lewy bodies and Lewy neurites whereas glial insoluble inclusions of aSyn are characteristic for multiple system atrophy (MSA) (1-3). In addition, mutations in the gene coding for the aSyn protein, SNCA, can cause familial PD with early onset, suggesting central involvement for aSyn in PD pathophysiology (4-8). aSyn is a natively unfolded brain protein that is mainly presynaptically localized but is found also in cytosol and nucleus. Proposed aSyn functions relate to synaptic neurotransmission, e.g. trafficking, packing and release of synaptic vesicles, but the exact physiological roles of aSyn still remain poorly understood (for review, see (9)).

Due to its inherently unfolded structure aSyn is prone to aggregation by various factors such as high concentration, posttranslational modifications, mutations, oxidative stress, low pH and metal ions. Moreover, formation of aSyn oligomers and fibrils leads to impairments in several cellular systems including mitochondria, ER-Golgi and other vesicular transport systems and the plasma membrane (10, 11). aSyn has three distinct domains in its protein structure: an N-terminal amphipathic region where familial PD mutations linked to aSyn aggregation are located, a hydrophopic central part (NAC) that is required for aSyn aggregation, and an acidic C-terminus that has been shown to regulate fibril formation (10). aSyn aggregation is a nucleation-dependent process, and it progresses from formation of misfolded soluble monomers and oligomers to protofibrils and mature fibrils with β-sheet structure and reduced solubility (10, 12). Although deposition of insoluble aSyn in Lewy bodies and neurites is the neuropathological hallmark of synucleinopathies, it appears that soluble oligomers and protofibrils are the most toxic aSyn species. In particular, dopaminergic neurons in the substantia nigra are highly sensitive to toxic aSyn species emphasizing the role of aSyn in PD pathology (10, 13).
Interactions of αSyn with proteins and lipids in its native cellular environment regulate its oligomerization and aggregation process. One mediator that enhances αSyn aggregation is prolyl oligopeptidase (PREP, POP, EC 3.4.21.26) (14). PREP is an 80-kDa protein with serine protease activity that is widely distributed among the species and can be found in the brain and various other tissues (15). It hydrolyzes peptides smaller than 30 amino acids, and substance P, angiotensin, thyrotropin-releasing hormone and vasopressin are the most widely studied PREP substrates (for review, see (16)). Several physiological functions for PREP have been proposed, including functions in cell proliferation and differentiation, inositol-1,4,5-triphosphate (IP3) signalling, and learning and memory (for review, see (17)). PREP activity has been reported to increase during aging and in neurodegenerative disorders, supporting the neuropeptide hypothesis (17, 18). Various small-molecule inhibitors of PREP enzyme activity have been developed but the results of PREP inhibition on neuropeptide levels in vivo and on memory and learning are unclear (19).

The size of αSyn protein is 140 amino acids, which makes it too large to be hydrolyzed by PREP and it has been proposed that PREP might serve as a nucleation point for αSyn aggregation (14, 20). In addition, PREP colocalizes with αSyn in the substantia nigra in human PD brains (21). PREP inhibition effectively prevents αSyn aggregation in a cell-free system, in αSyn-overexpressing cells and in transgenic mouse models (14, 22). Recently, we have identified PREP as a negative modulator of autophagosome formation, and treatment with PREP inhibitor, KYP-2047, induced macroautophagy and autophagosome formation via a beclin 1-dependent pathway (23). This was accompanied with increased clearance of high molecular weight αSyn species in the A30P transgenic mouse brain, suggesting PREP inhibition as a novel therapeutic strategy in synucleinopathies (23).

We hypothesized that modulation of αSyn aggregation by PREP may occur via multiple mechanisms, potentially also involving a direct protein-protein interaction. Although the effect of PREP on αSyn aggregation in a cell-free system (14) indirectly support this hypothesis, a direct interaction between αSyn and PREP has not been demonstrated. Here, we have used a combination of live-cell and cell-free methods to show that both wild-type PREP and a catalytically inactive mutant PREP with a serine-alanine mutation at residue 554 (S554A) interact with αSyn with low micromolar affinity. Both wild-type PREP and the S554A mutant enhance αSyn dimerization in cells; but only in cells expressing wild-type PREP is αSyn dimerization reduced by KYP-2047, a specific PREP inhibitor. Our data suggest that PREP modulates the early stage of αSyn aggregate formation, likely already at the dimerization stage, via a direct interaction with αSyn. These data provide further support for PREP as a nucleation point for αSyn aggregation, and provide mechanistic insight in how pharmacological inhibition of PREP reduces αSyn aggregation in cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals** – Chemicals used were purchased form Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Ethanol was purchased from Altia (Helsinki, Finland). Recombinant porcine PREP was produced in E. coli and purified as described in (24). Inactive PREP(S554A) mutant protein was obtained from Prof. Anne-Marie Lambeir (University of Antwerp, Belgium). The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-l-prolyl-2(S)-cyanopyrrolidine), was synthesized in the School of Pharmacy, University of Eastern Finland, as previously described (25). KYP-2047 was chosen as a reference compound since the biochemical and pharmacological data indicate that it is potent and selective, enters cells in culture and crosses the blood-brain barrier effectively in rodents (22, 24, 26, 27).

**DNA constructs** – The split *Gaussia princeps* luciferase (GLuc) expression plasmids used in this study were previously described in (28). The human αSyn cDNA used to clone αSyn-GLuc1/HA, αSyn(Δ118-140)-GLuc1/HA (with amino acid 118-140 removed), αSyn(A98-140)-GLuc1/HA (with amino acid 98-140 removed) and asyn-GLuc2/HA were obtained from ORFeome library (version 3.1, Genome Biology Unit, Institute of Biotechnology, University of Helsinki, accession number: BC013293), and was PCR-cloned in the Gluc vector with HA-tag using the KpnI-XhoI site (constructs graphically summarized in Fig. 2A and Fig. 4A). The PREP and PREP(S554A) plasmids used in this project were previously described in (23), and were used for cloning the PREP-GLuc2 and PREP(S554A)-GLuc2 constructs (graphically summarized in Fig. 3A). All GLuc constructs used in this study were previously described in (23, 28).
have the GLuc reporter fragment placed at the N-terminus separated by a (GGGGS)2SG linker.

Cell culture and transfection – Mouse Neuro-2A (N2A) neuroblastoma cells were used throughout the whole study. N2A cells were cultured in full Dulbecco’s Modified Eagle Medium (DMEM with additional 10% (v/v) FBS (Gibco, Invitrogen), 1% (v/v) L-Glutamine-Penicillin-Streptomycin solution (Lonza)) at 37°C, 5% CO2/water saturated air. Transfection of N2A was done using JetPei (Polyplus) according to manufacturer’s instruction.

Protein-fragment complementation assay (PCA) – PCA was performed as previously described in (28). N2A cells were plated on poly-L-Lysine coated 96-well plates (Perkin Elmer, white wall) at a density of 10,000 cells per well. 24 hours post-plating, reporter plasmids were transfected (125 ng of total plasmid DNA per well). PCA signal was read 24 hours post transfection. Cells were changed to phenol red-free DMEM (Gibco/Invitrogen) without serum 30 minutes before the measurement. GLuc PCA signal was detected by injecting 25 µl of native coelenterazine (Nanolight Technology) per well (final concentration of 20 µM), and the emitted luminescence was read by Varioskan Flash multiplate reader (Thermo Scientific). For each experimental condition, 4 replicate wells were performed. A 100 mM stock solution of KYP-2047 was prepared in DMSO, (final concentration of 20 µM), and the emitted signal generation. Quantitative analysis of the blot was done using QuantityOne software (Bio-Rad).

Cellular fractionation – Total cell lysate was prepared as described above in the Western Blot section. After 30-minute incubation on ice, each sample was adjusted to equal volume with the extraction buffer in poly-carbon centrifugation tubes (Beckman Coulter). Samples were centrifuged with a tabletop ultracentrifuge (Optima™ ultracentrifuge, Beckman Coulter) and TLA-100 rotor at 100 000 × g for 30 minutes. After centrifugation, each soluble fraction was collected into a separate tube, with protein concentration measured and loaded on gel for Western blot analysis. The insoluble pellet in each tube was washed once with milliQ-H2O, and mixed with 50 µl of Laemmli buffer (75 mM Tris-HCl with pH 6.8, 3% SDS, 15% glycerol, 3.75 mM EDTA, pH 7.4). The insoluble fraction samples were further sonicated using a rod sonicator (Labsonic® M.B. Braun Biotech International) for 2 × 1 second at 0.5 cycle and amplitude of 60% for solubilizing the pellets. Then, the insoluble protein fraction was solubilized in gel-loading buffer containing 0.25% mercaptoethanol, and loaded on gel for Western Blot analysis.

Native polyacrylamide gel electrophoresis (PAGE) – Native-PAGE was carried out as described earlier in (29) by leaving out SDS from the PAGE-gel, sample buffer (BioRad) and Tris-glycine running buffer. 6 µg of purified recombinant PREP or PREP(S554A) proteins were incubated with 30-fold molar excess of KYP-2047 or vehicle (0.15 % DMSO in PBS) for 30 minutes at room temperature. Samples were run on 10 % native gel containing a stacking gel. Gel was then fixed for 30 min in destaining buffer, (45% methanol, 10% acetic acid), stained with 0.1% Coomassie Brilliant Blue R-250 for 2 hours and destained 3-4 times for 30 minutes.

PREP activity assay – PREP activity assay was used to confirm the enzymatic activity of various PREP constructs, and it was performed as
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described in (22, 30). Briefly, the cells transfected with various PREP constructs were homogenized with lysis buffer (50 mM KH₂PO₄, 1.5 mM MgCl₂, 10 mM NaCl, 1 mM EDTA, pH 7.4) and the homogenates were centrifuged at 16,000 × g for 10 min at +4°C. The homogenate was preincubated with assay buffer for 30 min at 37°C. Then, the substrate (4 mM Suc-Gly-Pro-AMC) was added to initiate the reaction, and the incubation continued for 60 min at 37°C. The reaction was stopped with 1 M sodium acetate buffer (pH 4.2). The formation of 7-amino-4-methylcoumarin (AMC) was measured using a Wallac 1420 Victor fluorescence plate reader (PerkinElmer). The excitation and emission wavelengths were 360 and 460 nm, respectively. The protein concentration of the cell homogenate was determined using BCA protein assay kit (Thermo Scientific).

Microscale thermophoresis (MST) – MST was performed to study protein-protein interaction using purified recombinant proteins. Recombinant porcine PREP and PREP(S554A) proteins were labeled with the Monolith NT.115 protein labeling kit (NanoTemper Technologies, Germany) using red fluorescent dye NT-647 NHS (amine-reactive) according to the manufacturer’s instructions. Labeling reagents were removed by buffer-exchange column chromatography, and PREP and PREP(S554A) were eluted in PBS with 0.05% Tween-20. Binding assays were performed with a Monolith NT.115 Microscale Thermophoresis device using standard treated capillaries (both device and capillaries were from NanoTemper Technologies). In order to improve the accuracy of the K_d determination, while giving a fluorescence signal above 200 units, the concentration of labeled protein was kept minimum (range 1-100 nM) by diluting in assay buffer (final concentration of 0.05% Tween-20, 0.5 mg/ml BSA and 2.5 mM DTT in PBS) and modifying the led power between 20%-60%. Equal amount of labeled protein were titrated by purified recombinant human αSyn (Sigma, #S7820) in 1:1 series dilution starting from 35 µM, or with KYP-2047 in 10:1 and 1:1 series dilution starting from 10 µM. Curve fitting was done by NTanalysis software (NanoTemper Technologies) in the Thermophoresis + T-jump mode. Assimilated Curves generated from 3 replicate binding assays were analyzed in GraphPad Prism software to calculate the K_d values using non-linear regression and one site-specific binding with the Hill slope.

Surface Plasmon Resonance (SPR) - SPR sensor hydrogel was prepared according to the instructions of instrument manufacturer (BioNavis Ltd., Tampere, Finland). The immobilization of PREP protein was performed at 20 °C temperature, 20 µl/min flow rate and with 5 mM MES pH 5.0 as running buffer. PREP protein injection solution was 33 µg/ml in 5 mM pH 4.75 acetate buffer, prepared fresh from freezer aliquots immediately before injection. The immobilization cycle was as follows: The sensor surface was cleaned with 2 M NaCl + 10 mM NaOH cleaning solution. The hydrogel binding of PREP was tested with a pre-concentration injection. The surface was then cleaned again with the same cleaning solution. Then the surface was activated with EDC/NHS solution (of concentration 20/5 mg/ml) and PREP was injected in one of the flow channels, other flow channels remained as the reference channel. The surface was then deactivated with 1 M ethanolamine pH 8.5 solution. The immobilization resulted with 140-400 ng/mm² protein immobilized on the surface. The specificity of PREP sensor was tested with macroglobulin (1, 10 and 40 µg/ml at 30 µl/min) and bovine serum albumin (BSA) injections. No unspecific binding was detected.

Recombinant human αSyn (Sigma, #S7820) sample was run at 20 µl/min. The binding of α-synuclein was tested in logarithmic dilution 0.5, 5 and 50 µg/ml, which were diluted from a 500 µg/ml stock. The binding was analysed with TraceDrawer 1.3 for BioNavis (Ridgeview Instruments AB, Uppsala, Sweden) for binding constants. The unspecific binding of αSyn and PREP were tested with a macroglobulin sensor that was prepared in same manner as the PREP sensor, except that 50 µg/ml macroglobulin solution was used. This resulted in 100 ng/mm² protein immobilized on the sensor. No unspecific binding of αSyn or PREP was detected (data not shown).

Statistical analyses – Statistical analyses were carried out using student’s t-test (two groups) or one-way ANOVA with Bonferroni’s post-tests (three or more groups) in GraphPad Prism software. Significance was set at p < 0.05.

RESULTS

PREP overexpression increases αSyn reporter dimerization in N2A cells
PREP inhibition was previously shown to reduce chronic oxidative stress-induced accumulation of aSyn aggregates in SH-SY5Y neuroblastoma cells (22). In order to study the effects of PREP in the earliest stages of the aSyn aggregation process, we first developed an assay for studying aSyn oligomer formation in live cells. This system is based on protein-fragment complementation (PCA) where complementary fragments of Gausia princeps luciferase (GLuc) are attached to interacting proteins of interest and a luminescence signal is generated upon protein interaction in live cells (31). We first expressed the aSyn-GLuc1 and aSyn-GLuc2 PCA reporter constructs in cells together with wild-type PREP and PREP(S554A). In N2A neuroblastoma cells, which show no detectable endogenous PREP expression, there was a subtle increase in aSyn levels upon co-expression of PREP or PREP(S554A), a catalytically inactive mutant of PREP, with the aSyn-GLuc reporters (Fig. 1A-B) at 48 h post-transfection. We did not observe signs of aSyn cleavage in PREP expressing cells.

Next, we measured the PCA signal in N2A cells coexpressing the aSyn-GLuc reporters and PREP or PREP(S554A). As shown in Fig. 2B, increasing levels of PREP or PREP(S554A) in cells strongly promoted complementation with the maximal effects reaching 193% and 247% induction for PREP and PREP(S554A), respectively. Addition of PREP inhibitor KYP-2047 to the cells reduced PREP-induced aSyn complementation only in cells expressing the wild-type PREP but not in PREP(S554A) cells (Fig. 2C). KYP-2047 had no effect on aSyn reporter dimerization in control-transfected cells suggesting that the effect of KYP-2047 is PREP-specific and requires expression of catalytically active PREP (Fig. 2D).

To confirm that the cell-based assay system and selected time points reflect the early events in the aSyn-oligomerization process - not the formation of aSyn inclusions - we extracted cells with 1% Triton X-100 for cellular fractionation and analyzed Triton-soluble and insoluble fractions on Western blot. As shown in Fig. 2E, expression of PREP or PREP(S554A) or the treatment with KYP-2047 had no effect on aSyn solubility in N2A cells at 48 h post-transfection. This further supports that in N2A cells at these early time points, with no additional stress stimuli, the aSyn-GLuc reporters remain almost entirely in the soluble fraction.

Interactions of PREP and aSyn in live cells

To address the potential interaction of PREP with aSyn, we generated another PCA reporter, PREP-GLuc2, carrying a N-terminal GLuc2 tag (Fig. 3A). The expression and enzymatic activity of PREP-GLuc2 was assessed to confirm the functionality of the construct. Although the PREP-GLuc2 reporter is expressed at a lower level in N2A cells as compared to the untagged wild-type PREP (Fig. 3B), the PREP-GLuc2 reporter maintains hydrolytic activity towards a fluorescently labeled substrate peptide (Fig. 3C). As shown in Fig. 3D, expression of aSyn-GLuc1 or PREP-GLuc PCA reporters alone in cells generates only background luminescence signal. However, co-expression of PREP-GLuc2 or PREP(S554A)-GLuc2 reporters with aSyn-GLuc1 reporter results in generation of significant PCA signal suggesting that these proteins are found in close enough proximity (likely <10Å) in cells to allow refolding of the GLuc reporter fragments into an active luciferase reporter protein. Interestingly, PREP(S554A) shows more than 30% higher PCA signal with aSyn as compared to the wild-type PREP-GLuc2-expressing cells. We also verified the specificity of the aSyn-GLuc1 reporter-derived PCA signal with GLuc2-tagged GSAP(16K), a cytoplasmic protein that interacts with γ-secretase and the cytosolic domain of the amyloid precursor protein (APP)(32). GSAP(16k)-GLuc2 produced a significant PCA signal with the APP-C98-GLuc1 control but not with aSyn-GLuc1 (Fig. 3D).

Next, we tested the effect of KYP-2047 on the PREP-aSyn interaction. KYP-2047 binds to both PREP and PREP(S554A) (our MST data, not shown). Interestingly, KYP-2047 strongly increased the PREP-aSyn interaction (+207%) but had very little effect on PREP(S554A)-aSyn interaction (+109%) as compared to vehicle-treated cells expressing the same reporters (Fig. 3E).

Since the PCA data suggests that PREP and aSyn interact in cells, we sought to further characterize the interaction. PREP has hydrolytic specificity towards the C-terminal side of proline residues (33). The acidic 45-residue C-terminal domain of aSyn contains all five proline residues of the wild-type aSyn protein. We generated C-terminal truncation mutants of the aSyn-GLuc1 reporter for further interaction studies with PREP. The aSyn(Δ118-140) mutant lacks three and the aSyn(Δ98-140) mutant all five proline residues (Fig. 4A). Both truncation mutants are expressed in N2A cells, although at a slightly lower level.
lower level as compared to the full-length aSyn-GLuc1 reporter protein (Fig. 4B). When the C-terminal truncation mutants of aSyn-GLuc1 were co-expressed with the PREP-GLuc2 reporter, all the reporters generated comparable levels of PCA signals (Fig. 4C). Although the aSyn(Δ98-140)-GLuc1-PREP-GLuc2 reporter pair showed a 29% lower signal as compared to the full-length aSyn-GLuc1-PREP interaction, this could be explained by the somewhat lower level of aSyn(Δ98-140)-GLuc1 reporter protein expression (Fig. 4B). Altogether, the PCA data suggest that PREP and aSyn can interact in intact cells, and that neither the C-terminal proline residues of aSyn nor the enzyme activity of PREP are necessary for the interaction.

Direct interaction of PREP and aSyn in a cell-free system

In order to confirm a direct interaction of PREP and aSyn using purified proteins in a cell-free system, we turned to microscale thermophoresis (MST), a novel method for quantitative analysis of protein interactions in free solution (34, 35). Recombinant PREP and PREP(S554A) proteins were labeled with the red fluorescent dye NT-647. Addition of recombinant unlabeled aSyn protein to the MST capillaries containing wild-type PREP shows interaction between the two proteins, with a $K_d$ of 2.96 µM (Fig. 5A). In a similar MST experiment, recombinant PREP(S554A) also interacts with aSyn, with a $K_d$ value of 1.41 µM (Fig. 5B). Fluorescence time traces detected by the MST reader suggest low or no aggregation of the proteins during the experiments (insets in the Fig. 5A-B). This data confirms that there is a direct interaction between PREP and aSyn, with a binding affinity in the low micromolar range. Interestingly, similar to the PCA experiments, the cell-free MST experiments also suggest that the PREP(S554A) mutant has a slightly higher affinity for aSyn than the wild-type PREP.

The interaction between PREP and aSyn that was seen in MST was also confirmed with surface plasmon resonance (SPR). In the SPR experiments, the PREP sensor showed a clear signal with aSyn, and an affinity plot was created from three concentrations (Fig. 5C). The $K_d$ value of the interaction was 3.61 µM (average of three assays) that was similar to the $K_d$ value obtained from MST experiments. aSyn did not show any interaction with a macroglobulin sensor, and the PREP sensor did not react with macroglobulin or BSA, suggesting that the binding was specific. We also studied the interaction between PREP(S554A) and aSyn in SPR but despite repeated trials PREP(S554A)-aSyn interaction was not detected by this method.

KYP-2047 modifies conformational forms of wild-type but not inactive PREP mutant

It has been previously shown that KYP-2047 binding modifies conformation of PREP (29). Our data suggested that KYP-2047 binds both to PREP and PREP(S554A) but showed a decrease in aSyn reporter dimerization in PCA only when incubated with active PREP. We hypothesized that the wild-type and mutant PREP proteins may have differences in their conformational forms or KYP-2047 could modify them differently.

When wild-type PREP was applied on a native gel, three bands representing different conformational states were seen (Fig. 6). Incubation with KYP-2047 prior to gel application shifted wild-type PREP in a single faster migrating band, possibly representing monomeric PREP in a compact conformation (Fig. 6). Three bands were visible also for PREP(S554A) but the main form appears to migrate slightly faster as compared to wild-type PREP. Importantly, incubation with KYP-2047 has no effect on the three conformations of PREP(S554A) (Fig. 6). These data show that PREP can adopt multiple conformational states, which are sensitive to inhibitor binding. The differential sensitivity of wild-type PREP and the S554A mutant to inhibitor-induced conformation shifting offers a potential explanation why these PREP forms have different effects on aSyn dimerization in the presence of KYP-2047.
aggregation of aSyn in a cell-free model, the interaction between PREP and aSyn has not been documented earlier. In the current paper, we have now shown that PREP directly interacts with aSyn, increases its dimerization and that this effect can be countered with a specific PREP inhibitor, KYP-2047. aSyn is known to interact with a variety of proteins and small molecules, such as tyrosine hydroxylase and tau, metal ions and lipids (36-40). The N-terminal and central NAC domains of aSyn interact with lipids in cellular membranes and with some proteins (41-44) whereas the C-terminal domain shows low dimerization and with some proteins (45-48) does not dimerize in intact cells in its monomeric form. aSyn interacts with proteins, such as synphilin-1 (45) and tubulin (46), small molecules and macromolecules, such as dopamine (47), polysaccharides (48) and metal ions (11) can enhance aggregation of aSyn. Since the aggregation process is nucleation-dependent (12, 49, 50), it is possible that some of these interaction partners function as a seeding point for aSyn oligomerization and aggregation.

In this study, we have shown that PREP increases aSyn dimerization in intact cells in its native cellular environment, and that it directly interacts with aSyn in vitro. This suggests that PREP acts as a seed for aSyn dimerization and aggregation. PREP interacts with proteins and small molecules, such as dopamine (47), polysaccharides (48) and metal ions (11) can enhance aggregation of aSyn. Since the aggregation process is nucleation-dependent (12, 49, 50), it is possible that some of these interaction partners function as a seeding point for aSyn oligomerization and aggregation.

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aSyn aggregation (56). Using the C-terminal truncation mutants of aSyn, we showed that the proline-rich domain of aSyn is not required for PREP interaction. These data suggest that the PREP-interaction is determined mostly by the N-terminal and NAC domains of aSyn.

It appears that PREP has a dual effect on the aSyn aggregation process; it enhances the formation of soluble aSyn oligomers in a process that involves a direct PREP-aSyn interaction, and it also negatively affects autophagy – a catabolic pathway used for clearance of insoluble aSyn oligomers and fibrils (23). Importantly, a small-molecule PREP inhibitor affects both of these PREP actions that modulate accumulation of aSyn aggregates. Our current data shows that PREP inhibition reduces dimerization of aSyn via a direct protein-protein interaction. Moreover, PREP inhibition increases the clearance of aSyn aggregates by increasing autophagosome formation via beclin1 (23). Taken together, these results strongly support PREP inhibition as a potential new therapy for synucleinopathies.

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ABBREVIATIONS

The abbreviations used are: AMC, amino methyl coumarin; aSyn, \(\alpha\)-synuclein; GLuc, humanized Gaussia princeps luciferase; MST, microscale thermophoresis; NHS, N-hydroxysuccinimide; PCA, protein-fragment complementation assay; PD, Parkinson’s disease; PREP, prolyl oligopeptidase (a.k.a. prolyl endopeptidase); SPR, surface plasmon resonance; TBS, Tris-buffered saline.

FIGURE LEGENDS

Figure 1. Co-expression of PREP increases levels of aSyn in N2A cells. (A) The expression of PREP and PREP(S554A) together with GLuc-fragment- and HA-tagged aSyn’s was studied by Western blot. aSyn-GLuc1 and aSyn-GLuc2 were transfected at 0.75 \(\mu\)g plasmid DNA per well (each plasmid), while PREP and PREP(S554A) were transfected either at 0.75 \(\mu\)g or 1.5 \(\mu\)g. The total amount of plasmid DNA was set at 3 \(\mu\)g per well and was adjusted by mock plasmid when...
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Cells transfected with aSyn-GLuc1 and aSyn-GLuc2 plasmids only were used as the control. Blots were stained with antibodies to PREP, HA-tag (aSyn-GLuc1/2) and GAPDH as a loading control.

(B) Optical density-based quantification of aSyn levels in four replicate experiments (as in Fig. 1A). Both PREP and PREP(S554A) showed a trend towards increased aSyn levels but the difference did not reach significance. The average values are displayed as percent change as compared to control-transfected cells (mean ± SEM; n=4 independent experiments).

Figure 2. PREP co-expression promotes formation of soluble aSyn dimers in N2A cells.

(A) Schematic presentation of GLuc fragment-tagged aSyn reporter constructs used in this study.

(B) The effect of co-expression of PREP or PREP(S554A) with aSyn was studied by PCA. aSyn-GLuc1 and aSyn-GLuc2 were kept constant (31.25 ng plasmid DNA per well each) while increasing amounts of PREP or PREP(S554A) plasmid was transfected (12.5 ng, 37.5 ng and 62.5 ng). Total amount of plasmid was adjusted to 125 ng per well with mock plasmid when needed. Both PREP and catalytically inactive PREP(S554A) significantly increased the dimerization of aSyn with increasing levels of expression (one-way ANOVA; ***, p<0.001 PREP vs control). The average values are displayed as percent change as compared to control-transfected cells (mean ± SEM; n=4 independent experiments).

(C) The effect of KYP-2047 on aSyn dimerization in PREP-expressing cells was studied by PCA. Cells transfected with aSyn-GLuc1 and aSyn-GLuc2 (31.25 ng each), and PREP or PREP(S554A) (37.5 ng) were treated with at 1 µM, 5µM or 10 µM KYP-2047 for 4 hours. Both 5 and 10 µM doses of KYP-2047 significantly decreased aSyn dimerization when incubated with active PREP (one-way ANOVA; *, p<0.05 5 µM KYP-2047 vs control; **, p<0.01 10 µM KYP-2047 vs control) but KYP-2047 did not show any effect on aSyn dimerization in PREP(S554A)-expressing cells. DMSO was used as vehicle control. The average values are displayed as percent change as compared to control (mean ± SEM; n=4 independent experiments).

(D) The effect of KYP-2047 on aSyn dimerization without co-expression of PREP was studied by PCA. Cells transfected with aSyn-GLuc1 and aSyn-GLuc2 (31.25 ng each) were treated with 10 µM KYP-2047 for 4 hours. No change in aSyn dimerization was observed. DMSO was used as vehicle control. The average values are displayed as percent change as compared to control (mean ± SEM; n=4 independent experiments).

(E) Solubility of aSyn reporter proteins with co-expression of PREP or PREP(S554A) and KYP-2047 treatment was studied by cellular fractionation and Western blot. Cells transfected with aSyn-GLuc1 and aSyn-GLuc2 (0.75 µg each), PREP or PREP(S554A) plasmids were transfected at 1.5 µg. 5 µM KYP was added for 24 hours. No shift of aSyn reporters from Triton-soluble (S) to insoluble fractions (I) was observed. Blots were stained with antibodies to PREP, HA-tag (aSyn-GLuc1/2) and GAPDH as a loading control.

Figure 3. aSyn interacts with PREP and PREP(S554A) in live cells.

(A) Schematic presentation of GLuc fragment-tagged reporter constructs of aSyn and PREP used in this study.

(B) PREP protein expression after different construct transfections was studied by Western blot. All the constructs showed PREP protein expression, although PREP-GLuc2 was expressed at a lower level as compared to untagged PREP. Blot was stained with antibodies to PREP and GAPDH as a loading control.

(C) The enzymatic activity of PREP and PREP-GLuc2 was studied by an enzyme assay using a fluorescent substrate peptide. PREP activity in N2A cell lysates was significantly increased by PREP and PREP-GLuc2 transfections (one-way ANOVA; ***, p<0.001 PREP vs control; *, p<0.05 PREP-GLuc2 vs control) while cells transfected with PREP(S554A) or PREP(S554A)-GLuc2 did not show any enzymatic activity.

(D) The interaction of aSyn-GLuc1 with PREP-GLuc2 and PREP(S554A)-GLuc2 was studied by PCA. In order to confirm interaction specificity, the following reporter plasmids were co-expressed in pairs (62.5 ng each): PREP-GLuc2 with GLuc1 ("empty plasmid", no insert); PREP(S554A)-GLuc2 with GLuc1; aSyn-GLuc1 with GLuc2; aSyn-GLuc1 with GSAP(16k)-GLuc2; APPC98-GLuc1 with GSAP(16k)-GLuc2. In order to demonstrate the aSyn-PREP interaction, aSyn-GLuc1 was co-
expressed with PREP-GLuc2 or PREP(S554A)-GLuc2 (62.5 ng each). Both PREP-GLuc2 and PREP(S554A)-GLuc2 showed a significant PCA signal with aSyn-GLuc1 (one-way ANOVA; ***, \( P<0.001 \)), and interestingly, PREP(S554A)-GLuc2 had even stronger interaction with aSyn-GLuc1 than active PREP (one-way ANOVA; ***, \( P<0.001 \)). The average values are displayed as direct luminescence readout. Signals below 700 relative luminescence units (RLU) were regarded as background noise (mean ± SEM; \( n=3 \) independent experiments).

(E) The effect of KYP-2047 on aSyn-PREP and aSyn-PREP(S554A) interaction was studied by PCA. Same transfections were used as in Fig. 3B but in addition the cells were treated with 5 µM KYP-2047 for 4 hours. KYP-2047 showed a significant increase in the PCA signal of PREP-GLuc2 and aSyn-GLuc2 (one-way ANOVA; *, \( P<0.05 \)), but not with inactive PREP(S554A). DMSO was used as vehicle control. The average values are displayed as percent change as compared to the individual controls for aSyn-PREP and aSyn-PREP(S554A), respectively (mean ± SEM; \( n=3 \) independent experiments).

Figure 4. aSyn-PREP interaction does not require the proline-rich C-terminal domain of aSyn.

(A) Schematic presentation of GLuc fragment-tagged aSyn constructs with different C-terminal truncations used in this study. P indicates a proline residue in the C-terminal tail of aSyn.

(B) The expression aSyn-GLuc1, aSyn(Δ118-140)-GLuc1 and aSyn(ΔC98-140)-GLuc1 was analyzed by Western blot. Each plasmid was transfected at 1.5 µg per well. All the constructs were appropriately expressed in N2A cells as determined by the anti-HA staining of the Western blot. GAPDH served as a loading control.

(C) The effect of C-terminal truncation of aSyn on aSyn-PREP interaction was studied by PCA. Co-expression of PREP-GLuc2 with aSyn(ΔC118-140)-GLuc1 showed similar PCA signal as the full-length aSyn-GLuc1 but co-expression of PREP-GLuc2 with aSyn(ΔC98-140)-GLuc1 showed a decrease in PCA signal. However, this may be due to the lower expression level of aSyn(Δ98-140) as compared to the full-length aSyn-GLuc1 reporter (Fig. 4B). The average values are displayed as percent change as compared to the control (mean ± SEM; \( n=3 \) independent experiments). * indicates significant difference with \( P<0.05 \) (one-way ANOVA).

Figure 5. Microscale thermophoresis and surface plasmon resonance confirm a direct interaction between PREP and aSyn.

The interactions between aSyn and PREP (A), and between aSyn and PREP(S554A) (B) were studied by MST. Equal amounts of fluorescently labeled (NT-647) recombinant PREP or PREP(S554A) were titrated by recombinant unlabeled aSyn at various concentrations. Similar to PCA results, aSyn binds to PREP and PREP(S554A) with a \( K_d \) of ~2.96 µM and ~1.41 µM, respectively. Fluorescence time traces taken from single experiment suggest low or no aggregation had occurred in both sets of experiments (insets in both graphs). The curve and \( K_d \) values were calculated by averaging \( K_d \) curves assimilated using NTanalysis software from three independent experiments (mean ± SEM).

(C) A time versus signal plot of SPR measurement is shown, normalized by Bmax of the 1st order analysis. Three doses of aSyn were used in the injections (0.5, 5 and 50 µg/ml). \( K_d \) determined by SPR was 3.61 µM and it was the mean of three independent experiments.

Figure 6. KYP-2047 alters the conformational forms of active PREP but not PREP(S554A).

Recombinant PREP and PREP(S554A) proteins on native-PAGE revealed three different conformations, which likely represent different open/close monomeric and oligomeric forms of the protein. However, inhibition of PREP with KYP-2047 shifts the slower-migrating conformations into a single band, likely representing closed monomeric conformation of PREP. PREP(S554A) had similarly three conformations but they were not affected by KYP-2047.
Figure 1.
Figure 2.
Figure 4.
Figure 5.
Figure 6.
Prolyl Oligopeptidase Enhances α-synuclein Dimerization via Direct Protein-Protein Interaction
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