terminal region (1–60) of α-synuclein is involved in the dimerization and exifone binding. This is in contrast with a previous report by Norris *et al.*, in which they suggested that dopamine inhibited the aggregation of α-synuclein by binding to the C-terminal residues 125–129 (i.e., YEMPS) and stabilizing the soluble oligomers.[6] The discrepancy might be due to the fact that they analyzed the dopamine-binding sites by using deletion mutants lacking the C-terminal regions and did not use full-length α-synucleins.

High-resolution NMR spectra of inhibitor-bound α-synuclein monomer and dimer

In order to characterize the behavior of α-synuclein monomer and dimer formed in the presence of polyphenolic compounds, we conducted a structural analysis of inhibitor-bound α-synuclein monomer and dimer using ultra-high-field NMR spectroscopy. NMR signals of backbone amides constitute excellent probes to provide maps of the interacting sites and to examine the effects of modifications.[13] Fig. 6a and b shows the ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectra of uniformly ^{15}N-labeled Exi-monomer and Exi-dimer, as well as control monomer, recorded at a proton observation frequency of 920 MHz. The amide resonances of Exi-monomer and Exi-dimer were assigned by comparing the NMR spectral data with those of control α-synuclein monomer. Little chemical shift difference was detected between Exi-monomer and control monomer for most observed peaks, except for the signals corresponding

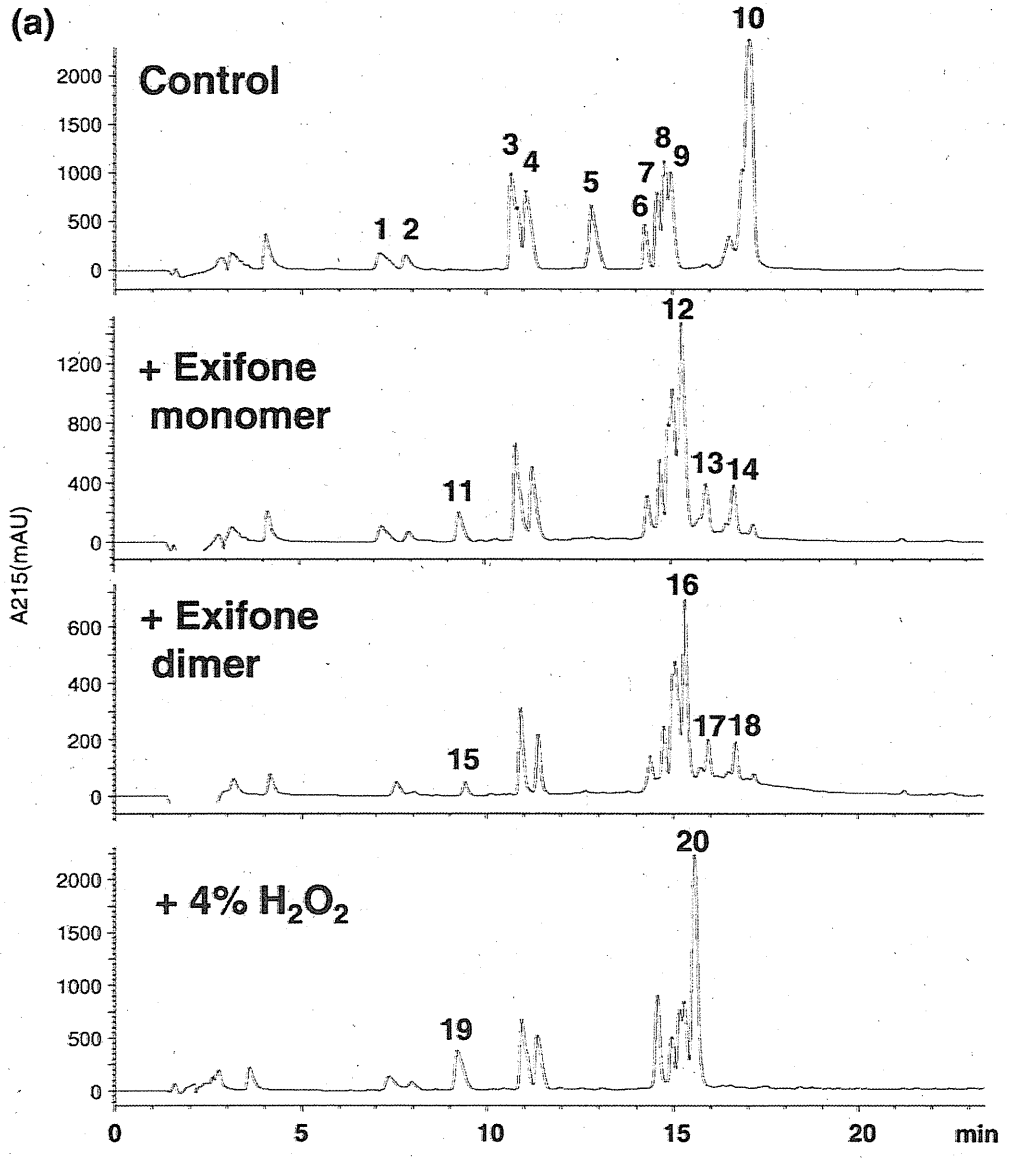

Fig. 3. Tryptic peptide mapping and MS analysis of α-synucleins. (a) Reverse-phase HPLC patterns of monomeric α-synuclein (control), Exi-monomer, Exi-dimer, and H_2O_2-treated α-synuclein monomer. (b) Observed masses and peak assignments of the peptides separated on a reverse-phase column. Oxidation of methionine residues was observed in Exi-monomer and Exi-dimer, as well as H_2O_2-treated α-synuclein monomer.

(b)

Peak No.	$(M+H)^+$ Observed	$(M+H)^+$ Calculated	Assignment
1	830.6 873.5 1072.6	830.4 873.4 1072.5	QGVAEAAGK (24-32) EGVVAAAEK (13-21) AKEGVVAAAEK (11-21)
2	1180.7 1295.8 1524.8	1180.6 1295.6 1524.8	TKEGVLYVGSK (33-43) EGVVHGVATVAEK (46-58) TKEGVVHGVATVAEK (44-58)
3	951.5	951.5	EGVLYVGSK (35-43)
4	1295.7	1295.6	EGVVHGVATVAEK (46-58)
5	770.5	770.3	MDVFMK (1-6)
6	1606.6	1606.8	TVEGAGSIAAATGFVKK (81-97)
7	2157.2	2157.1	TKEQVTNVGGAVVTGVTAVAQK (58-80)
8	1478.5	1478.7	TVEGAGSIAAATGFVK (81-96)
9	1928.0	1928.0	EQVTNVGGAVVTGVTAVAQK (61-80)
10	4295.0	4286.7	NEEGAPQEGILEDMPVDPDNAYEMPSEEGYQDYEPEA (103-140)
11	803.0	802.3.0	M*DVFM*K (1-6) (M*:methionine sulfoxide)
12	4322.0	4318.7	NEEGAPQEGILEDM*PVDPDNAYEM*PSEEGYQDYEPEA (103-140)
13	4311.0	4302.7	NEEGAPQEGILEDM#PVDPDNAYEM#PSEEGYQDYEPEA (103-140) (One of two methionine residues (M#) was oxidized)
14	4312.0	4302.7	NEEGAPQEGILEDM#PVDPDNAYEM#PSEEGYQDYEPEA (103-140)
15	802.0	802.3	M*DVFM*K (1-6)
16	4322.0	4318.7	NEEGAPQEGILEDM*PVDPDNAYEM*PSEEGYQDYEPEA (103-140)
17	4311.0	4302.7	NEEGAPQEGILEDM#PVDPDNAYEM#PSEEGYQDYEPEA (103-140)
18	4312.0	4302.7	NEEGAPQEGILEDM#PVDPDNAYEM#PSEEGYQDYEPEA (103-140)
19	803.0	802.3	M*DVFM*K (1-6)
20	4322.0	4318.7	NEEGAPQEGILEDM*PVDPDNAYEM*PSEEGYQDYEPEA (103-140)

Fig. 3 (*legend on previous page*)

to Met5, Met116, Met127, and their neighboring residues (Fig 6a). The observed chemical shift differences are mostly attributable to the oxidation of methionine residues. The differences in peak intensities between Exi-monomer and control monomer were also generally small (Fig. 6c). These results indicate that the dynamical features of both synuclein monomers are almost the same, and methionine oxidation itself does not greatly influence the structural characteristics of α-synuclein.

It is noteworthy that significant reductions in signal intensity [I(Exi-dimer)/I(control monomer)< 0.8] were observed for the peaks originating from the N-terminal region (1–60) of Exi-dimer compared with the control monomer (Fig. 6d). This result shows that the N-terminal regions are involved in exifone-induced dimerization of α-synuclein, in accordance with the results obtained from Asp-N digestion of the Exi-dimer. The gradual reduction in the signal intensities might be explained by heterogeneous dimerization around the N-terminus. The observed reduction in signal intensity, in our case, was not due to chemical exchange between the inhibitor-free and inhibitor-bound states of α-synuclein, as had been suggested by Rao *et al.*,[10] because the inhibitor-induced dimer and monomer were each purified to homogeneity and free or exchangeable inhibitors were removed by gel-filtration column chromatography and buffer exchange. Similar NMR spectra were observed for dopamine- and gossypetin-induced dimers (Supplementary Fig. S4), indicating that the N-terminal dimerization modes induced by dopamine, exifone, and gossypetin are the same or at least very similar. On the other hand, the C-terminal portion of exifone-bound dimer was still predominantly random coil in character, as observed in the control monomeric α-synuclein. These

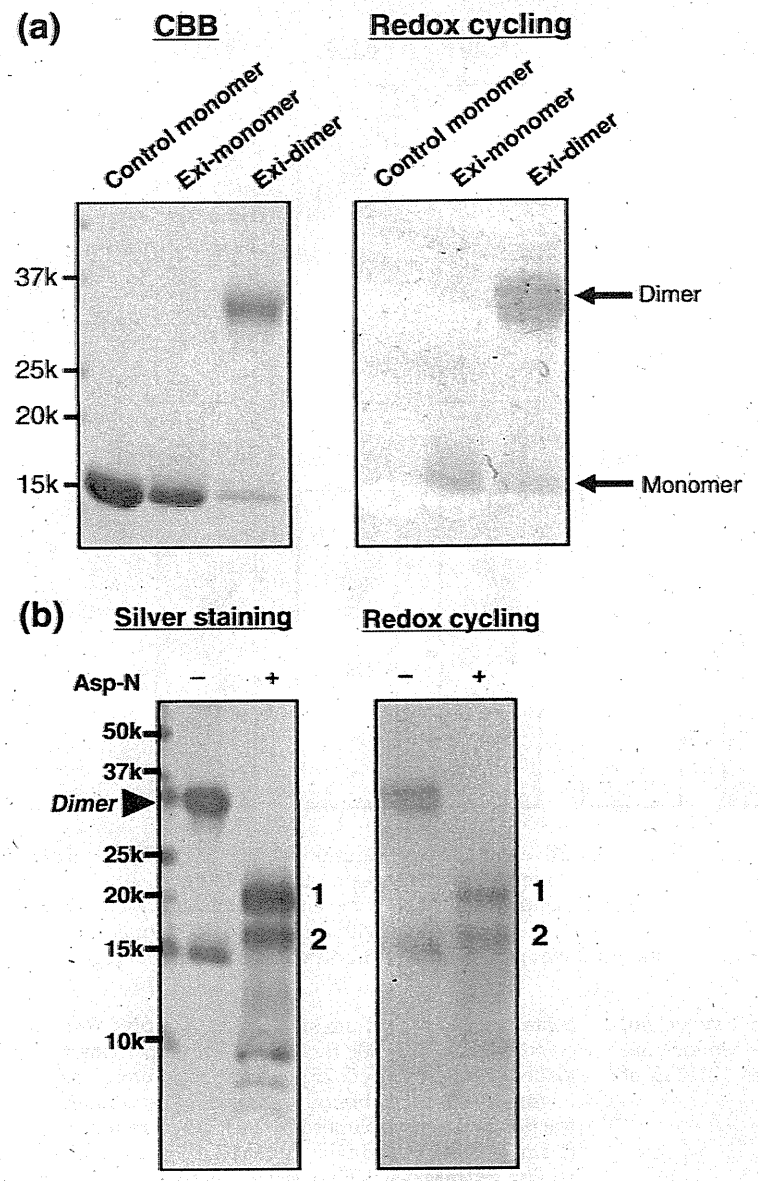

Fig. 4. Detection of exifone bound to α-synucleins by redox-cycling staining. (a) Control monomer, Exi-monomer, and Exi-dimer were stained with Coomassie brilliant blue (left) or by redox cycling (right). Exifone-bound α-synucleins were stained by redox cycling, appearing as purple-blue bands in the Exi-monomer and Exi-dimer lanes, due to NBT reduction to formazan (right). (b) Asp-N digestion of Exi-dimer. Exi-dimer was digested with endoproteinase Asp-N and the fragments were detected by silver staining (left) and redox staining (right). Two major bands corresponding to 20 kDa and 16 kDa (nos. 1 and 2, respectively) were positive for redox-cycling staining.

observations indicate the importance of the N-terminal region in α-synuclein assembly.

It is of note that three missense mutations in familial PD (A30P, E46K, and A53T) are located in the N-terminal region of α-synuclein. Recent NMR analyses suggest that these mutations may be altering the physicochemical properties of the protein, such as net charge (E46K) and secondary-structure propensity (A30P and A53T).[19] The binding of exifone, gossypetin, or dopamine to α-synuclein might also alter the net charge and/or secondary-structure propensity.

We did not observe the colloidal formation of exifone, gossypetin, or dopamine by electron microscopy (data not shown) as reported by Feng *et al.*[5] The discrepancy might be due to differences in the compounds used or differences in the proteins investigated. The inhibition mechanism of these three compounds seems rather specific because the N-terminal region was specifically involved in inhibitor binding, which is in contrast to the nonspecific colloidal inhibition.

In summary, we have characterized the inhibitor-bound α-synuclein dimer and showed that the N-terminal region (1–60) plays a key role in dimerization and inhibitor binding. Further studies are under way in our laboratory to elucidate the mechanisms of inhibitor-induced oligomer formation at atomic resolution.

Materials and Methods

Antibodies

Polyclonal antibodies were raised against synthetic peptides corresponding to residues 1–10, 11–20, 21–30, 31–40, 41–50, 51–60, 61–70, 75–91, and 131–140 of human α-synucleins, prepared as described previously.[12] Antibody

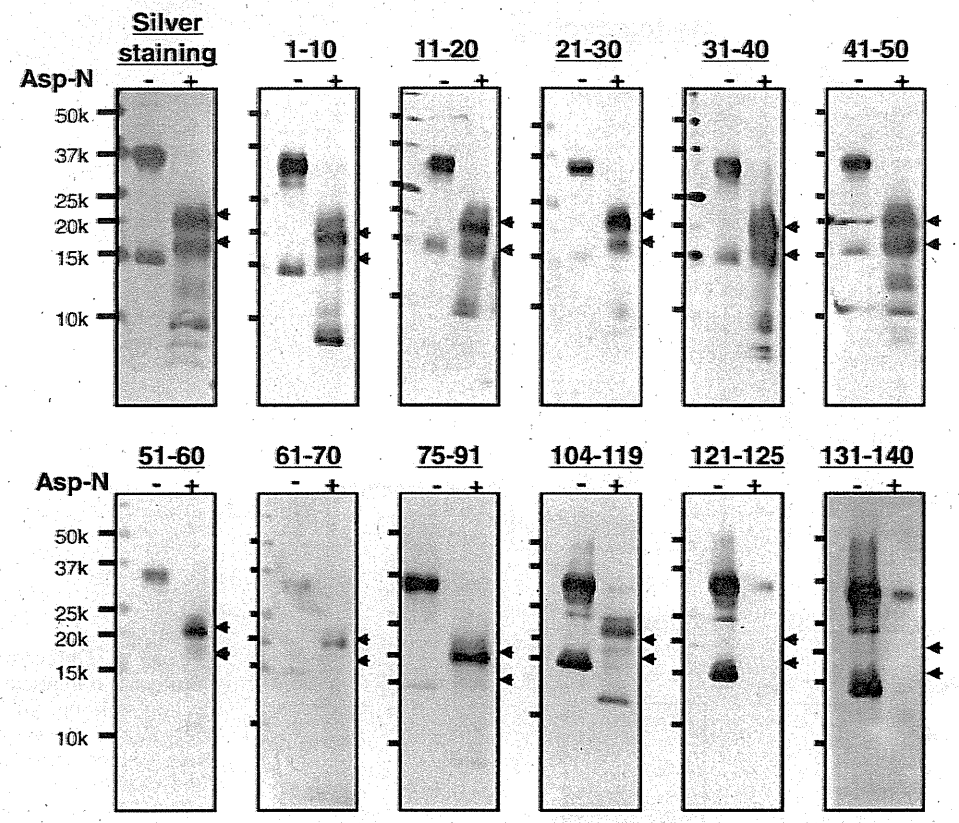

Fig. 5. Immunoblot analysis of Asp-N digests of Exi-dimer. Silver staining and immunoblots of Asp-N digests of Exi-dimer with a panel of anti-α-synuclein antibodies raised against nine peptides (corresponding to residues 1–10, 11–20, 21–30, 31–40, 41–50, 51–60, 61–70, 75–91, and 131–140).[12] Experimental details are given in Materials and Methods. Two major fragments (band nos. 1 and 2) were detected with silver staining (indicated with arrowheads). Fragment no. 1 was positive for antibodies to the N-terminal region, 1–97, and no. 2 was positive for antibodies to the N-terminal region, 1–50.

Syn259, which recognizes residues 104–119 of α-synuclein, was kindly provided by Dr. S. Nakajo. Monoclonal antibody Syn211, which recognizes residues 121–125 of α-synuclein, was purchased from Zymed.

Protein expression and purification

Expression of isotopically labeled α-synuclein was performed as described.[13] Human α-synuclein cDNA in bacterial expression plasmid pRK172 was used for production of isotopically labeled protein for NMR analyses.[20] Codon 136 was changed from TAC to TAT by site-directed mutagenesis to avoid cysteine misincorporation.[21] Uniformly ^{15}N-labeled α-synuclein was expressed in *Escherichia coli* BL21(DE3) cells grown in M9 minimal medium containing 1 g/L [^{15}N]NH_4Cl, while unlabeled α-synuclein was expressed using LB medium. Cell lysates were subjected to boiling and subsequently to ammonium sulfate precipitation. The precipitated α-synuclein was extensively dialyzed against 20 mM Tris–HCl (pH 8.0) and then purified with DEAE ion-exchange chromatography.

Preparation of inhibitor-bound α-synuclein monomers and dimers

Purified ^{15}N-labeled recombinant α-synuclein (9 mg/mL) was incubated with 2 mM inhibitor (exifone, gossypetin, or dopamine; see Fig. 1) for 30 days at 37 °C in 30 mM Tris–HCl containing 0.1% sodium azide. The samples were then centrifuged at 113,000*g* for 20 min. The supernatants were loaded on a Sephadex G-25 gel-filtration column to separate oligomers from unbound inhibitor. The eluates were fractionated on a Superdex 200 gel-filtration column (1 cm×30 cm), eluted with 10 mM Tris–HCl (pH 7.5) containing 150 mM NaCl. Eluates were monitored at 215 nm. α-Synuclein monomer and dimer fractions were each concentrated and the concentrates were subjected to NMR analysis. Protein concentrations were determined using HPLC and bicinchoninic acid protein assay kit (Pierce).

Mass spectrometry

Samples were spotted on a sample plate and mixed with the matrix solutions, saturated sinapic acid (Fluka) or α-cyano-4-hydroxycinnamic acid (Fluka) in 50% acetonitrile/H_2O containing 0.1% (v/v) trifluoroacetic acid. Mass spectra were obtained by MALDI-TOF MS using a Voyager-DE Pro mass spectrometer (PerSeptive Biosystems).

Peptide mapping of H_2O_2-treated and inhibitor-bound α-synucleins

Inhibitor-bound α-synuclein monomer and dimer were prepared as described above. For methionine oxidation, α-synuclein monomer (7 mg/mL) was incubated with 0–4% H_2O_2 at room temperature for 20 min and then dialyzed

against 30 mM Tris–HCl (pH 7.5) to remove H_2O_2. To identify the modification, inhibitor-bound α-synuclein monomer and dimer, as well as H_2O_2-treated α-synuclein, were incubated with trypsin at 37 °C for 18 h at an enzyme-to-substrate ratio of 1:50 (mol/mol) in 30 mM Tris–HCl (pH 7.5). Digested peptide products were separated by reverse-phase HPLC on a Supersphere Select B column (2.1×125 mm; Merck) and analyzed by MALDI-TOF MS.

Determination of stoichiometry of exifone/α-synuclein complexes

The stoichiometry of exifone/α-synuclein complexes was determined by measuring the absorbance of exifone at 385 nm using a spectrophotometer (UV-1600 PC, Shimadzu Co). Exifone-bound monomeric and dimeric α-synucleins were isolated by gel-filtration chromatography as described above.

Redox-cycle staining

Samples were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were incubated in 0.24 mM NBT (Sigma), 2 M potassium glycinate solution (pH 10.0) in the dark for 16 h at room temperature and then dipped in 100 mM sodium borate (pH 10.0). Exifone-bound α-synuclein was specifically stained as purple-blue bands due to NBT reduction to formazan.

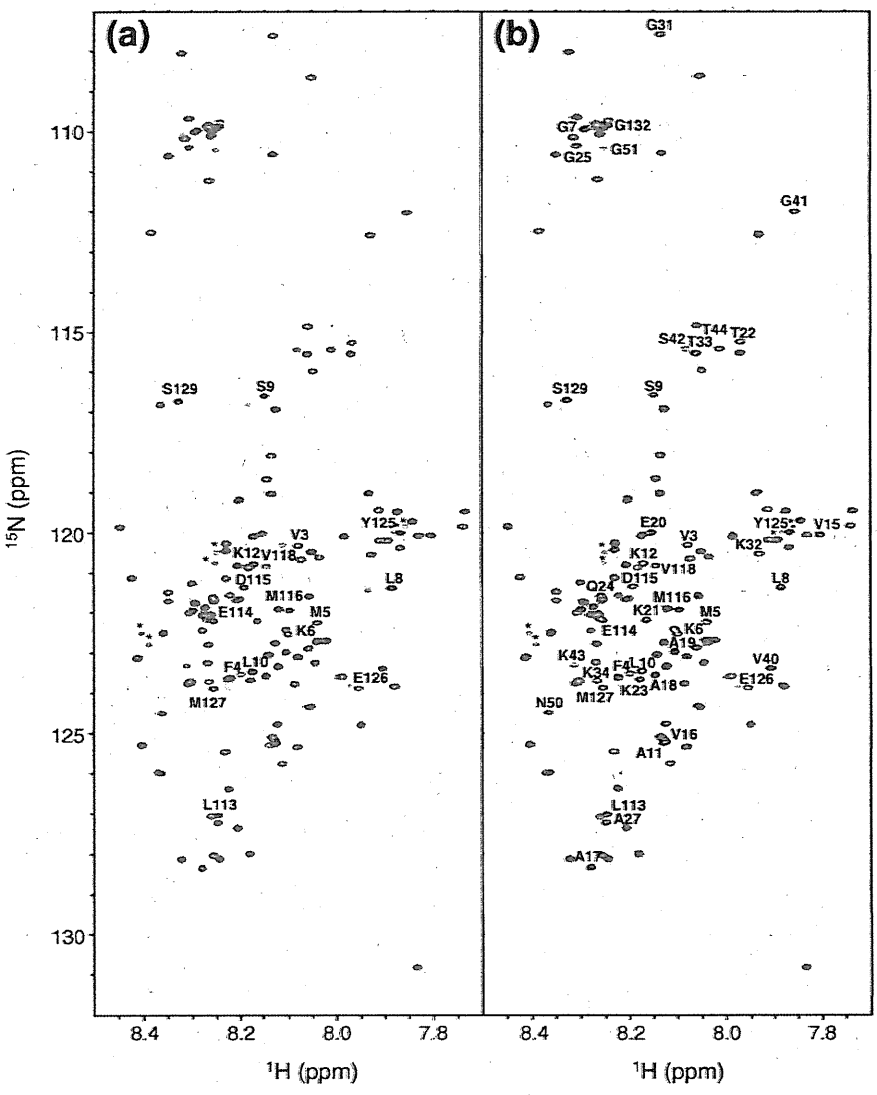

Fig. 6. NMR spectral comparison of exifone-bound ^{15}N-labeled α-synuclein dimer and control monomer. (a) ^{1}H–^{15}N HSQC spectra of ^{15}N-labeled Exi-monomer (red) and ^{15}N-labeled control monomer (black) recorded at a proton frequency of 920 MHz. (b) ^{1}H–^{15}N HSQC spectra of ^{15}N-labeled Exi-dimer (red) and ^{15}N-labeled control monomer (black). (c) Plot of the relative peak intensities, *I*(Exi-monomer)/*I*(monomer), of the HSQC cross-peaks in the Exi-monomer and control monomer *versus* the amino acid sequence of α-synuclein. (d) *I*(Exi-dimer)/*I*(monomer) of the HSQC cross-peaks in the Exi-dimer and control monomer. Signals derived from oxidized methionines and their neighboring residues (indicated with asterisks in a and b) were split and not taken into account. The peak splittings mostly reflect a mixture of *R* and *S* isomers of methionine sulfoxide.[18]

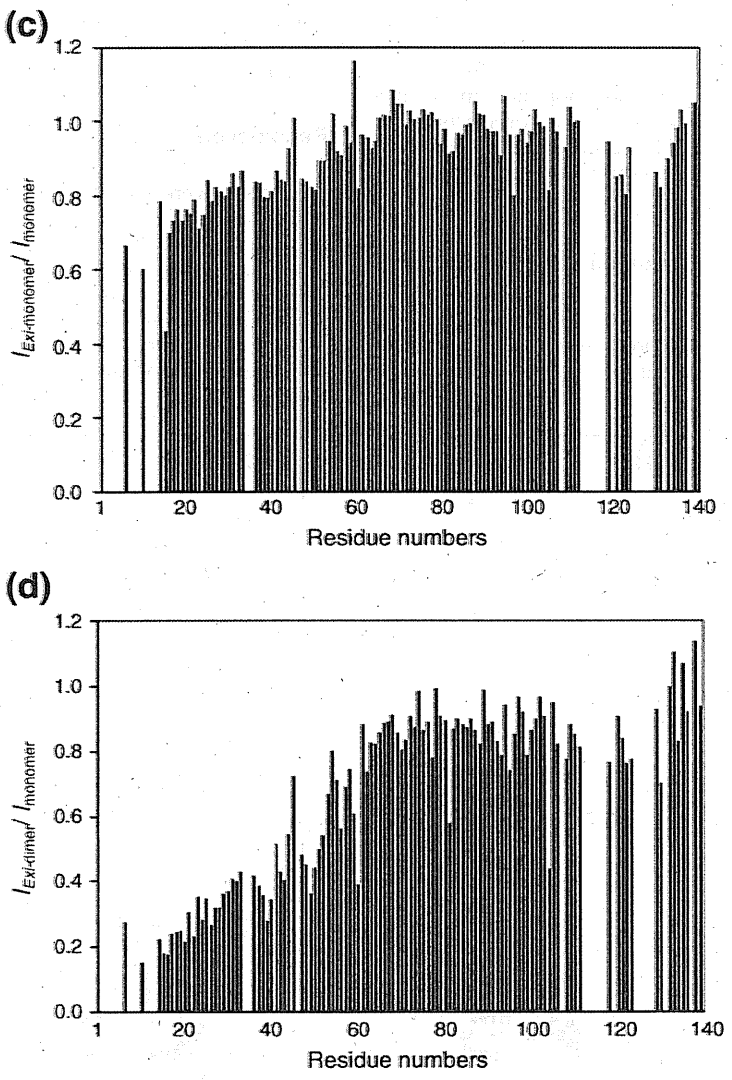

Fig. 6 *(legend on previous page)*

Asp-N digestion of α-synuclein dimer

α-Synuclein dimer (0.25 mg/mL) in 30 mM Tris–HCl (pH 7.5) was treated with 40 μg/mL of Asp-N (Roche) at 37 °C for 1 h. The reaction was stopped by adding 2× SDS sample buffer [4% SDS, 0.16 M Tris–HCl (pH 6.8), 30% glycerol] and the solution was boiled for 5 min. The samples were loaded onto 15% Tris/tricine SDS-PAGE gel, and the digested products were detected by silver staining (kit from Wako), immunoblotting, and redox-cycling staining. For immunoblotting, SDS-PAGE gels were blotted onto polyvinylidene fluoride membranes, blocked with 3% gelatin/phosphate-buffered saline, and incubated overnight at room temperature with anti-α-synuclein antibody in 10% FBS/phosphate-buffered saline. After washing, the blots were incubated for 2 h at room temperature with biotinylated secondary antibody (1:500) (Vector Laboratories). Following further washing, the blots were incubated with peroxidase-labeled avidin–biotin (Vector laboratories) for 30 min at room temperature and developed with $NiCl_2$-enhanced diaminobenzidine (Sigma).

NMR measurements

The samples for NMR experiments were prepared at a concentration of 0.1–1.0 mM in 90% H_2O/10% D_2O (v/v), 10 mM sodium phosphate buffer, and 100 mM NaCl at pH 7.0. NMR experiments were performed at 10 °C using a JEOL JNM-ECA920 spectrometer equipped with a 5-mm triple resonance probe. Backbone assignments of α-synuclein monomer were achieved by means of standard triple resonance experiments, as described previously.[13] The samples were checked by SDS-PAGE before and after NMR measurements, and it was confirmed that aggregation of inhibitor-bound α-synuclein monomer and dimer did not occur under these conditions. NMR time domain data were processed with the nmrPipe package[22] and the spectra were analyzed by using

Sparky software (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

Acknowledgements

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.10.068

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The FASEB Journal • Research Communication

Phosphorylated α-synuclein can be detected in blood plasma and is potentially a useful biomarker for Parkinson's disease

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ABSTRACT **Parkinson's disease (PD) is characterized by the presence of Lewy bodies containing phosphorylated and aggregated α-synuclein (α-syn). α-Syn is present in human body fluids, including blood plasma, and is a potential biomarker for PD. Immunoassays for total and oligomeric forms of both normal and phosphorylated (at Ser-129) α-syn have been used to assay plasma samples from a longitudinal cohort of 32 patients with PD (sampled at mo 0, 1, 2, 3), as well as single plasma samples from a group of 30 healthy control participants. The levels of α-syn in plasma varied greatly between individuals, but were remarkably consistent over time within the same individual with PD. The mean level of phospho-α-syn was found to be higher (P=0.053) in the PD samples than the controls, whereas this was not the case for total α-syn (P=0.244), oligo-α-syn (P=0.221), or oligo-phospho-α-syn (P=0.181). Immunoblots of plasma revealed bands (at 21, 24, and 50–60 kDa) corresponding to phosphorylated α-syn. Thus, phosphorylated α-syn can be detected in blood plasma and shows more promise as a diagnostic marker than the nonphosphorylated protein. Longitudinal studies undertaken over a more extended time period will be required to determine whether α-syn can act as a marker of disease progression.—Foulds, P. G., Mitchell, J. D., Parker, A., Turner, R., Green, G., Diggle, P., Hasegawa, M., Taylor, M., Mann, D., Allsop, D. Phosphorylated α-synuclein can be detected in blood plasma and is potentially a useful biomarker for Parkinson's disease. *FASEB J.* 25, 4127–4137 (2011). www.fasebj.org**

Key Words: Lewy body · oligomer · immunoassay · immunoblot

PARKINSON'S DISEASE (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD) and is characterized clinically by the 3 cardinal motor symptoms of resting tremor, rigidity, and bradykinesia. Patients often exhibit further symptoms, including postural imbalance, gait disturbance, and a mask-like facial expression. In the advanced stages of PD, nonmotor symptoms can also appear, including anxiety, depression, dementia, and psychosis. The defining neuropathological features of idiopathic PD are the loss of dopaminergic neurons from the substantia nigra (SN) and the presence of Lewy bodies (LBs) and Lewy neurites (LNs) in surviving neurons of this and other brain regions (1). Similar lesions are present within the cerebral cortex in the related disorder of dementia with Lewy bodies (DLB; ref. 2). LBs and LNs contain a misfolded, fibrillar, and phosphorylated form of the protein α-synuclein (α-syn; refs. 1, 3). Pathological changes involving α-syn, chiefly in glial cells, also occur in multiple system atrophy (MSA), and, therefore, PD, DLB, and MSA are often collectively referred to as "α-syncleinopathies" (2). Duplication (4, 5), triplication (6), and mutation (7–9) of the gene encoding α-syn (*SNCA*) are all causes of hereditary forms of either PD or DLB. α-Syn oligomers are believed to be toxic to cells, as are oligomers derived from the various proteins associated with several other protein-misfolding neurodegenerative disorders, such as AD or the prion disorders (10, 11). Overexpression of wild-type or mutant α-syn in animal models can produce a phenotype resembling PD, including SN degeneration, movement problems and responsiveness to L-dopa therapy (12–15). Together, these observations suggest that α-syn plays a pivotal role in the development of the α-synucleinopathies (16).

PD is one of several neurological movement disor-

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ders that can produce similar symptoms, and a correct diagnosis is critically dependent on clinical examination to rule out disorders that can mimic PD. A diagnosis of PD is considered if the person exhibits more than one of the 3 cardinal motor symptoms mentioned above (17). The presence of resting tremor supports the diagnosis of PD more than the other two symptoms, but ~20% of patients with autopsy-confirmed PD fail to develop any resting tremor (18). Moreover, only 69–70% of people with autopsy-confirmed PD have at least two of the cardinal signs of the disease and 20–25% of people with two of these symptoms have a pathological diagnosis other than PD (19, 20). Perhaps even more surprising is the finding that 13–19% of people who demonstrate all three of the cardinal features have a pathological diagnosis other than PD (19, 20). Because the progression of neurological movement disorders and their treatment varies greatly, proper clinical diagnosis is essential for correct patient management. Furthermore, by the time PD is diagnosed, >60% of dopaminergic neurons in the SN can already be lost (21), making accurate early diagnosis, ideally before clinical symptoms appear, essential for any effective neuroprotective intervention strategy. Also, the clinical diagnosis of early PD may be difficult because although the patient might complain of symptoms suggesting PD, the neurological examination may be normal (22). These problems with clinical diagnosis have led to an increased interest in the development of diagnostic markers for PD, including advanced brain imaging methodologies (23) and molecular biomarkers (24). Genetic testing for mutations in genes linked to familial PD (including *SNCA*) is available (25), but it is only relevant when there is a strong family history, or when symptoms present at an unusually young age.

We have reported that α-syn is released from cells and is present in human body fluids, including cerebrospinal fluid (CSF) and blood plasma (26). This extracellular form of α-syn seems to be secreted from neuronal cells by exocytosis (27, 28) and could play an important role in cell-to-cell transfer of α-syn pathology in the brain (29). There is now an emerging consensus that the levels of α-syn are, on average, lower in samples of CSF taken from a group of patients with PD compared with a group of normal or neurological controls (30, 31), especially when the confounding variables of age and blood contamination are taken into account (32, 33). However, obtaining CSF is an invasive procedure, and analysis of α-syn levels in CSF is not generally amenable to longitudinal study. There are also some studies of α-syn as a potential biomarker in the much more accessible peripheral blood, with an initial report suggesting increased levels of this protein in plasma samples from patients with PD compared with those from healthy controls (34). However, subsequent studies have reported decreased levels of α-syn in PD plasma (35) or no significant change (36). We have shown that the levels of oligomeric α-syn appear to be significantly elevated in plasma samples from a group of patients with PD compared with a group of diseased controls (37). To develop this line of enquiry further, we have now set up a longitudinal study in newly diagnosed patients with PD to examine the levels of various different forms of α-syn, including phosphorylated and/or oligomeric forms, in blood plasma. Because α-syn accumulates in a phosphorylated and aggregated form in LBs (3), it is possible that these modified, pathological forms of the protein will more accurately reflect the fundamental neuropathology of PD than straightforward measures of "total" α-syn (33, 38). Our ultimate aim is to develop a relatively simple test for the early diagnosis of PD, or a surrogate marker for monitoring the progression of PD. Here, we report the results obtained during the initial phase of this longitudinal study.

MATERIALS AND METHODS

Patient population and clinical method

Participants for this study were recruited (with ethical approval, using appropriate consenting procedures) from the neurological service based at the Royal Preston Hospital along with other similar departments in the northwest of England. The diagnosis of PD was based on the UK Parkinson's Disease Society diagnostic criteria for PD (39). Severity of disease was defined in terms of patients satisfying the criteria for stages 1 or 2 on the Hoehn and Yahr scale.

The overall target for the study was to follow a cohort of 200 patients meeting these criteria over a period of 2–3 yr, reviewing them at 4- to 6-mo intervals. This study is ongoing, but the plan was also to follow the first 32 patients more intensively over the initial phases of the study, and this group was seen at monthly intervals for the first 3 mo. The results from these 32 patients over the first 3 mo are presented here. Blood samples were obtained from the participating sites (Preston and Arrowe Park). Around 3 ml of blood was collected in tubes containing EDTA, and the plasma was separated within 3 h by centrifuging the blood at 3000 *g* for 10 min. The plasma was immediately stored at −80°C. Appropriate care was taken to avoid contamination of the plasma samples with cells or components of the pellet obtained from the centrifugation. The samples were thawed at room temperature directly before analysis. Repeated freeze/thaw cycles were avoided.

Control subjects were healthy individuals with no apparent neurological or known psychiatric symptoms who were the spouses of patients attending the Cerebral Function Unit clinics at Hope Hospital (Salford Royal Hospital, National Health Service Foundation Trust) for investigation and diagnosis of dementia. These control subjects were recruited as part of an ongoing investigation into the genetics and molecular biology of dementia approved by the Oldham Local Research Ethics Committee. Blood plasma was prepared and stored as described above.

Preparation of recombinant α-syn

Recombinant α-syn (without any purification tag) was prepared at Lancaster University from *Escherichia coli* using the following protocol. pJEK2 was used to transform FB850, a *rec* A^- derivative of BL21 (DES) pLysS. FB850 carrying this plasmid was grown in an 800-ml batch culture, and protein expression was induced through the addition of isopropyl-β-

D-thiogalactopyranoside (IPTG). A protein with a molecular weight of ~17 kDa started to accumulate in the cells 30 min after induction and reached maximum levels after 150 min. Immunoblot analysis identified this protein as α-syn using an anti-α-syn mouse monoclonal antibody (MAb 211; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After a 3-h induction, the suspension was centrifuged, and the cells were resuspended in buffer. The cells were lysed by sonication, and then cell debris and insoluble material were removed by centrifugation at 4°C for 1 h at 30,000 rpm. α-Syn was extracted from the supernatant by ammonium sulfate precipitation, then purified using two chromatography columns; mono Q and Superdex 200 (Amersham Biosciences, Piscataway, NJ, USA). After purification, 5 μg of protein ran as a single band when observed on a Coomassie blue-stained SDS gel, corresponding to monomeric α-syn.

Preparation of phosphorylated recombinant α-syn

Phosphorylated α-syn was prepared from recombinant α-syn, as described previously (40). Briefly, α-syn (630 μM) was incubated with casein kinase II (CK2; New England Biolabs, Ipswitch, MA, USA) in 1 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM ATP at 30°C for 24 h. For effective phosphorylation, CK2 was added to the reaction mixture at 2-h intervals for the first 10 h (7500 U×5). The reaction was stopped by boiling for 5 min, cleared by centrifugation at 113,000 *g* for 20 min at 4°C, and then loaded onto a Resource Q anion-exchange column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 8.0) and 0.2 M NaCl, and then eluted with a linear gradient of NaCl from 0.20 to 0.35 M for 15 min at a flow rate of 1 ml/min. Fractionated samples were analyzed by immunoblotting with a phosphorylation-dependent anti-α-syn antibody, PSer129 (Epitomics, Burlingame, CA, USA), and mass spectrometry. The PSer129-positive phosphorylated α-syn recovered in the fractions with ~0.3 M NaCl was concentrated by ammonium sulfate precipitation.

Preparation of oligomeric forms of recombinant α-syn

To prepare a standard for the oligomeric α-syn immunoassay, the recombinant protein was oligomerized by incubation at 45 μM in PBS in an orbital shaker at 37°C for 5 d, and the monomer and oligomer were separated by size-exclusion chromatography. A sample (0.5 ml) of preaggregated α-syn was loaded onto a Superdex 200 column (44×1 cm) connected to a fast protein liquid chromatography (FPLC) system (Atka Purifier; GE Healthcare, New York, NY, USA) and eluted with running buffer (PBS) at a flow rate of 0.5 ml/min. Absorbance of the eluate was monitored at 280 nm; fractions of 1 ml were collected, and protein concentration was determined.

To prepare a standard for the oligo-phospho-α-syn immunoassay, the phosphorylated protein was oligomerized by incubation at 50 μM in PBS in an orbital shaker at 37°C for 5 d. Aggregation of the protein was confirmed by thioflavin T assay. In this case, the amount of sample available was too small to fractionate by size-exclusion chromatography.

Immunoassay methods

We have already established immunoassay methods for the measurement of total and soluble oligomeric forms of α-syn in human biological fluids, including blood plasma (37, 41), and these methods have been further optimized.

Total α-syn

A 96-well microtiter plate (Iwaki, Holliston, MA, USA) was coated with 100 μl/well of anti-α-syn monoclonal antibody 211 diluted 1:1000 (0.2 μg/ml; Santa Cruz Biotechnology) in 50 mM $NaHCO_3$ (pH 9.6) and incubated at 4°C overnight. The wells were then washed 4 times with PBS containing 0.05% Tween-20 (PBS-T) and were incubated for 2 h at 37°C with 200 μl/well of freshly prepared blocking buffer (2.5% gelatin in PBS-T). The plate was washed again 4 times with PBS-T, and 100 μl of the assay standard or plasma samples was added to each well (each plasma sample was diluted 1:40 with PBS), and the assays were performed in triplicate. Following this, the plate was incubated at 37°C for 2 h. After a repeat washing with PBS-T, 100 μl/well of the detection antibody, anti-α/β/γ-synuclein FL-140 (Santa Cruz Biotechnology), dilution 1:750 (0.27 μg/ml) in blocking buffer was added, and the plate was incubated at 37°C for 2 h. After another wash with PBS-T, the plate was incubated with 100 μl/well of secondary antibody [goat anti-rabbit horseradish peroxidase (HRP); Sigma, St. Louis, MO, USA], dilution 1:10,000 in blocking buffer at 37°C for 2 h. The plate was then washed again with PBS-T before adding 100 μl/well of Sure Blue TMB microwell peroxidase substrate (KPL, Gaithersburg, MD, USA) and leaving the color to develop for 30 min at room temperature. Finally 100 μl/well of stop solution (0.3 M H_2SO_4) was added, and absorbance at 450 nm was determined. Recombinant monomeric α-syn was used to create a standard curve.

Oligomeric α-syn (oligo-α-syn)

The microtiter plate was coated and blocked using the same method as the assay for total α-syn. The wells were then washed 4 times with PBS-T, and 100 μl of the plasma sample (diluted 1:25 with PBS) or assay standard (oligo-α-syn) was added to each well, in triplicate. Following this, the plate was incubated at 37°C for 2 h. After a repeat wash with PBS-T, 100 μl/well of the detection antibody, biotinylated anti-α-synuclein 211 (diluted 1:1000 in blocking buffer) was added, and the plate was incubated at 37°C for 2 h. After another wash with PBS-T, the plate was incubated with 100 μl/well of streptavidin-europium, diluted 1:500 in streptavidin-europium buffer (Perkin Elmer, Wellesley, MA, USA) and shaken for 10 min. After a further 50-min agitation on a rotating platform, the plate was washed again with PBS-T, before adding 100 μl/well enhancer solution (Perkin Elmer). Finally, the plates were read on a Wallac Victor^2 1420 multilabel plate reader (Perkin Elmer), using the time-resolved fluorescence setting for europium.

Total phosphorylated α-syn (pS-α-syn)

The antibody-sandwich ELISA for total α-syn was modified to detect only the protein phosphorylated at Ser-129 by replacing the 211 phospho-independent capture antibody with polyclonal anti-α-synuclein N-19 (Santa Cruz Biotechnology), diluted 1:3,000 (0.07 μg/ml). The phospho-dependent rabbit monoclonal antibody, Phospho (pS129) antibody (Epitomics), used at a dilution of 1:3000, was the chosen detection antibody. This antibody detects only α-syn phosphorylated at Ser-129. The preferred secondary antibody was human serum absorbed goat anti-rabbit HRP, 1:3000 (KPL), rehydrated in 1 ml H_2O. Recombinant pS-α-syn was used as the assay standard.

Oligomeric phosphorylated α-syn (oligo-pS-α-syn)

The antibody-sandwich immunoassay for oligo-α-syn was modified to detect only phosphorylated, oligomeric forms of the

protein, by replacing the 211 phospho-independent capture antibody with the phospho-dependent rabbit monoclonal antibody, pS129 (Epitomics), used at a dilution of 1:3000. The detection antibody was biotinylated pS129 at a dilution of 1:400. Recombinant oligo-pS-α-syn was used to generate a standard curve.

Preparation of biotinylated antibodies

To prepare the biotinylated antibody, 200 g Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) was reacted with the required antibody (1 ml, 200 μg/ml) in PBS and then placed on ice for 2 h. The mixture was desalted on Bio-Spin-6 columns (Bio-Rad, Richmond, CA, USA) to remove excess uncoupled biotin and the biotinylated antibody was stored at −20°C until use.

Immunocapture of α-syn from plasma

Dynabeads covalently coupled with recombinant protein G were derivatized with goat polyclonal anti-α-syn synuclein N-19 antibody (Santa Cruz Biotechnology), as recommended by the manufacturer (Dynal Biotech, Wirral, UK). Plasma (500 μl) was added to the beads and incubated overnight at 4°C. The plasma samples were chosen according to the immunoassay results, with one sample giving a high signal for the phosphorylated protein, the other a low signal, and a control containing PBS only. The beads were then washed 3 times with 0.1 M phosphate buffer (pH 8.2). Any captured protein was eluted from the beads by boiling for 10 min in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) and was examined by gel electrophoresis and immunoblotting.

Gel electrophoresis and immunoblotting

The protein eluted from the magnetic Dynabeads was separated on 12.5% acylamide gels. The separated proteins were transferred to nitrocellulose membranes (0.45 μm, Invitrogen) at 30 V, 125 mA for 1 h. Membranes were blocked with 5% dried skimmed milk dissolved in PBS-Tween (PBST), for 1 h. The membranes were probed overnight at 4°C with either phospho-dependent rabbit anti-α-synuclein monoclonal antibody pS129 (Epitomics) at a dilution of 1:5000; rabbit polyclonal anti-synuclein antibody FL-140 (Santa Cruz Biotechnology) at a dilution of 1:1000 (0.2 μg/ml); or rabbit anti-ubiquitin antibody FL-76 (Santa Cruz Biotechnology) at a dilution of 1:1000 (0.2 μg/ml) in PBST. The membranes were washed 3 times in PBST, followed by incubation with human serum absorbed HRP-conjugated goat anti-rabbit (Sigma), 1:10,000 in PBST, for 1 h. The protein bands were visualized using ECL reagents (Pierce), as described by the manufacturer.

Analysis of immunoassay data

A set of standards for one of the 4 different assays (*i.e.*, for total α-syn, oligo-α-syn, pS-α-syn, or oligo-pS-α-syn) was included on each microtiter plate, as appropriate for the type of protein being measured on that plate. Standard curves were fitted using nonlinear least squares (see **Fig. 1** for representative examples of standard curves for each of the four different immunoassays). The samples of blood plasma from

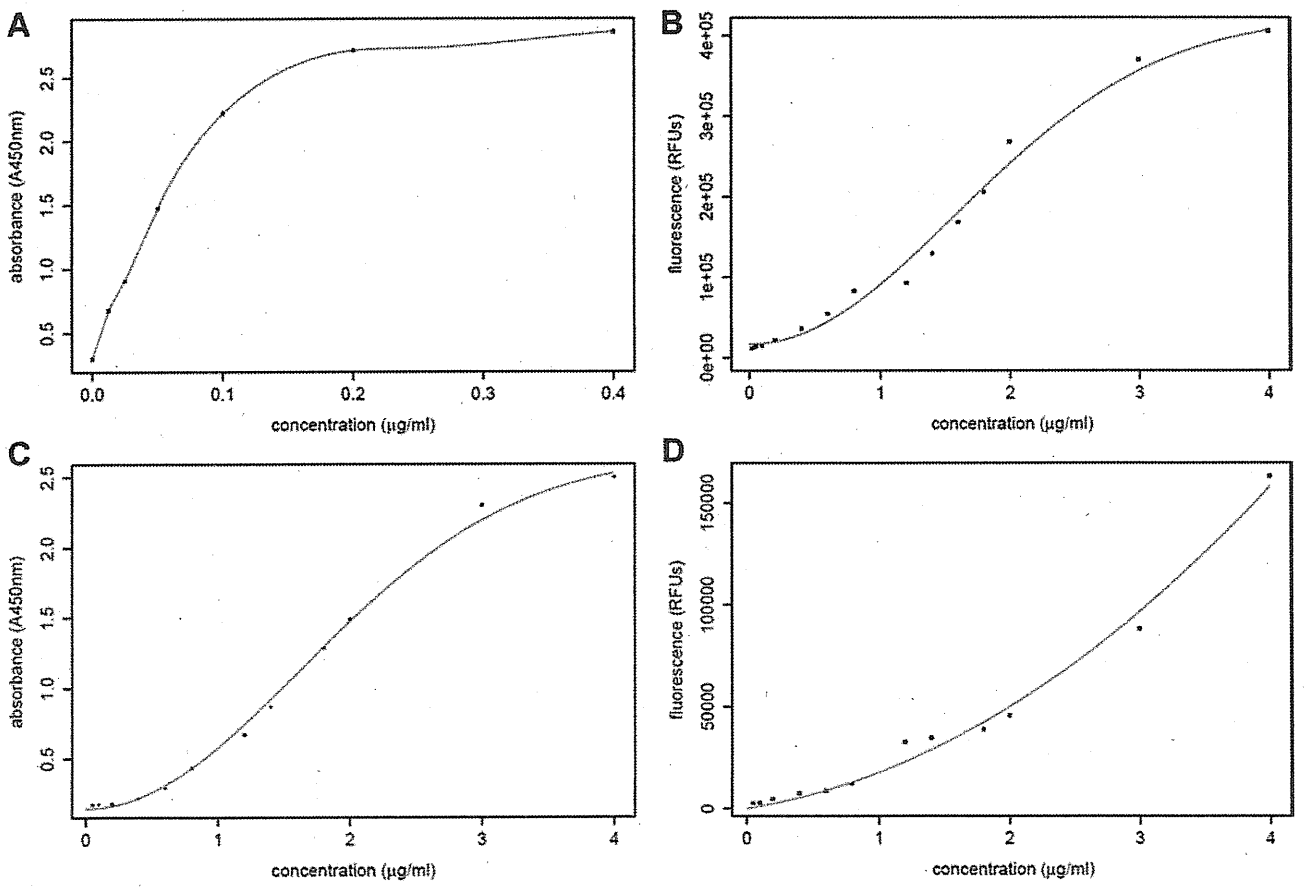

Figure 1. Examples of standard curves obtained for total α-syn (*A*), oligo-α-syn (*B*), pS-α-syn (*C*) and oligo-pS-α-syn (*D*). These are representative curves, each obtained from a single ELISA plate.

patients with PD and controls, diluted as indicated above, were measured in triplicate for each individual at each time point. The standard curves for each individual plate were used to transform the absorbance values (total α-syn, pS-α-syn) or relative fluorescence units (RFU; oligo-α-syn, oligo-pS-α-syn) for that particular plate into protein concentrations, and, in this way, any variation between plates was accounted for. The specificity of the oligo-α-syn immunoassay toward aggregated forms of α-syn has been reported previously (37, 41) but was confirmed here by analysis of fractions obtained by gel filtration of preaggregated, recombinant α-syn; only the peak containing α-syn oligomers, and not the monomer peak, was detected by the oligo-α-syn immunoassay. Also, as expected, the nonphosphorylated form of α-syn gave no signal in the pS-α-syn immunoassay, and the oligo-pS-α-syn immunoassay detected only pS-α-syn that had been preaggregated (data not shown). Further, when the blood plasma samples were immunodepleted with anti-α-syn antibodies C211 or FL-140, each coupled to magnetic Dynabeads, and then tested in the immunoassays, only trace signals could be detected above background compared to the nonimmunodepleted samples (data not shown).

To investigate whether the protein levels changed over time (*i.e.*, during the first 3 mo) a linear mixed model was fitted to the longitudinal data from each assay (details in Supplemental Data).

A classic 2-sample *t* test was used to determine whether there was any significant difference between the mean levels of each of the different forms of α-syn when comparing the plasma samples from the patients with PD with those from the healthy controls. To better satisfy the assumptions underlying this test, the empirical distributions were constructed on the logarithmic scale to obtain a more symmetric distribution than was obtained on the original scale.

RESULTS

Patient population and demographics

Demographic details of the cohort of 32 patients with PD that was followed at monthly intervals for 3 mo are summarized in **Table 1**. The mean age of this cohort on ascertainment and initial sampling was 68.2 yr (youngest 56 yr, oldest 85 yr). Among the 30 recruited healthy controls, there were 13 males and 17 females, with a median age of 63 yr and mean age of 61.5 yr (youngest 42 yr, oldest 75 yr). The PD case and control subjects were recruited in parallel, at the same clinical centers, and the blood samples were taken and processed by the same personnel at each site. Moreover, the plasma samples were effectively randomized for analysis, with both control and PD samples being assayed together on the same microtiter plates.

TABLE 1. *Demographic details of the cohort of 32 patients with PD*

Parameter	Value
Gender (male/female)	23/9
Hoehn and Yahr 1.0	5
Hoehn and Yahr 1.5	3
Hoehn and Yahr 2.0	24
Median PD onset age (yr)	61.9 (55.5–69.7)
Age at study recruitment (yr)	68.4 (62.3–73.8)
Disease duration at study recruitment (yr)	4.9 (3.1–9.3)

Values in parentheses indicate interquartile range.

Longitudinal data from patients with PD

Figure 2 presents a bar plot of the total α-syn plasma concentrations for each individual with PD over time (*i.e.*, for mo 0, 1, 2, 3) where, within each time point, we have averaged over triplicate measurements. It can be seen that the levels of total α-syn varied greatly between individuals, within an overall range of 0.01–6 μg/ml. Although a few individuals did show small, stepwise increases or decreases of total α-syn levels over this (very short) sampling period (see, for example, patient 32 in Fig. 2*B*), one of the most striking findings from this study was that, overall, the immunoassay results from the repeat PD plasma samples were remarkably consistent within each individual over time. This was a general finding for the results from all four of the different α-syn immunoassays, but it is illustrated here (Fig. 2) for the total-α-syn data only. Data for the other 3 assays (Supplemental File S2), together with a linear mixed model-based analysis (Supplemental File S1) for all 4 α-syn assays, are available in Supplemental Data. From the latter analysis, it is clear that the variation in α-syn levels across time within an individual is negligible relative to the variation across individuals. The model specifies a time trend, in addition to accounting for inherent differences in protein levels between individuals and differences across time within an individual. In all cases, the confidence interval for the estimated temporal effect covered 0. Thus, we conclude that there was no significant change over time for the levels of α-syn being measured by any of the immunoassays.

Comparison of patients with PD and controls

Empirical distributions of the α-syn concentrations for each assay were highly skewed on the original scale. **Figure 3** presents box plots pertaining to each assay, stratified according to patients with PD and controls. Note that the whiskers of the box plots extend by no more than the range of the data (largest minus smallest value) multiplied by the interquartile range. Extending the whiskers to the largest and smallest values would yield a rather compressed box. An apparent feature of the box plots is that the median concentration of α-syn for the patients exceeds that of the controls for both of the assays for phosphorylated α-syn (*i.e.*, pS-α-syn in Fig. 3*B*, and oligo-pS-α-syn in Fig. 3*D*). The reverse is true regarding the nonphosphorylated assays (total-α-syn in Fig. 3*A* and oligo-α-syn in Fig. 3*C*). Further, the interquartile range (*i.e.*, box height) reports that the concentrations are far less dispersed for controls compared to patients for both of the phosphorylated α-syn assays. For the nonphosphorylated assays, the controls display a larger spread of concentrations.

To investigate the potential of α-syn as a means of

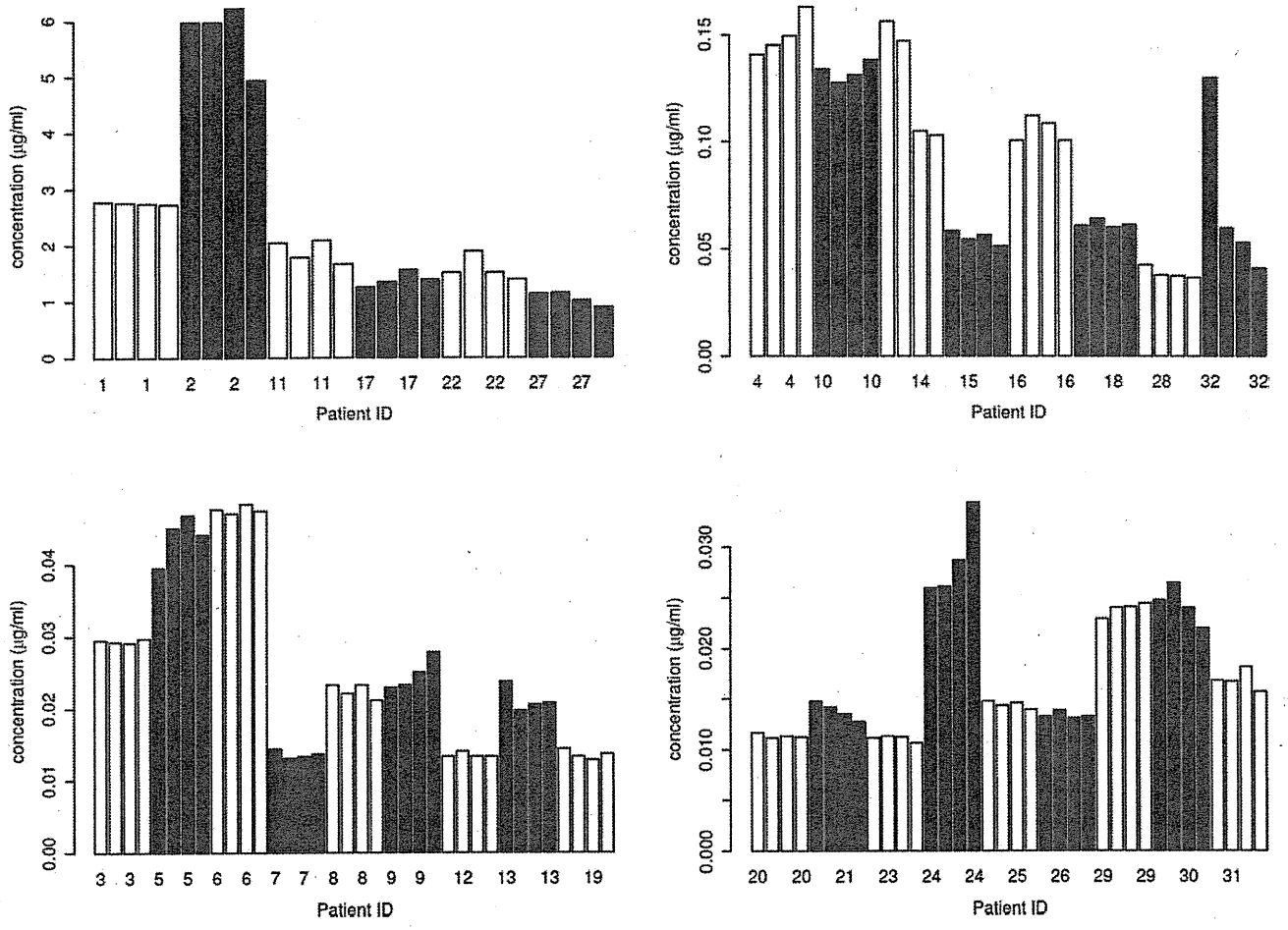

Figure 2. Longitudinal data for the levels of total α-syn in plasma samples from all of the 32 patients with PD. Consecutive bars for each patient represent the level of total α-syn in blood plasma samples taken at 0, 1, 2 and 3 mo. The participants are assigned to one of the 4 sections depending on their overall levels of protein.

discriminating between patients with PD and controls, we determined whether there was any significant difference between the average level of α-syn (on the logarithmic scale) across patients and controls, within all 4 α-syn assays. Because there was no consistent change in α-syn levels over time, nor across replicates within time, in the plasma samples from patients with PD, the concentrations for mo 0, 1, 2 and 3 were averaged over time and replicates in order to obtain a single mean value for each individual patient. Under a classical two-sample *t* test, the mean level of pS-α-syn was found to be marginally significantly higher for the patients than for the healthy controls (P=0.053). On the other hand, there was no difference across the average levels of patients and controls with regard to total α-syn (P=0.244), oligo-α-syn (P=0.221), or oligo-pS-α-syn (P=0.181).

Association with gender and age

The levels of α-syn showed no association with gender. For the total and oligo-α-syn assays, sampling age was a marginally significant −0.049 (−0.099, −0.001; P=0.052) and significant −0.009 (−0.019, −0.001; P=0.045) predictor, respectively, of α-syn levels in the patients with PD. On the other hand, the P values corresponding to the effect of sampling age on α-syn levels in the patients, under the pS-α-syn and oligo-pS-α-syn assays, were 0.412 and 0.274, respectively. The levels of α-syn showed no correlation with age in the control group. We did also analyze the data adjusting for age, but found no significant effects, and the adjustments did not materially change the (lack of) significance of the relevant assay results. Therefore, we chose to report the unadjusted results for simplicity.

Receiver operating curve (ROC) analysis

Figure 4 displays an ROC curve constructed to evaluate the utility of plasma pS-α-syn levels in discriminating patients with PD from healthy controls. The area under the curve (AUC) of 0.68 suggests that pS-α-syn has some potential value as a discriminant between patients and controls. AUC curves for 2 of the other 3 assays gave AUC values of less than 0.5 (0.28 for total α-syn and 0.22 for oligo-α-syn), which would also indicate a potentially informative result, with plasma levels of these being lower in patients than in controls. An AUC of 0.62 for oligo-pS-α-syn, however, suggests that in this

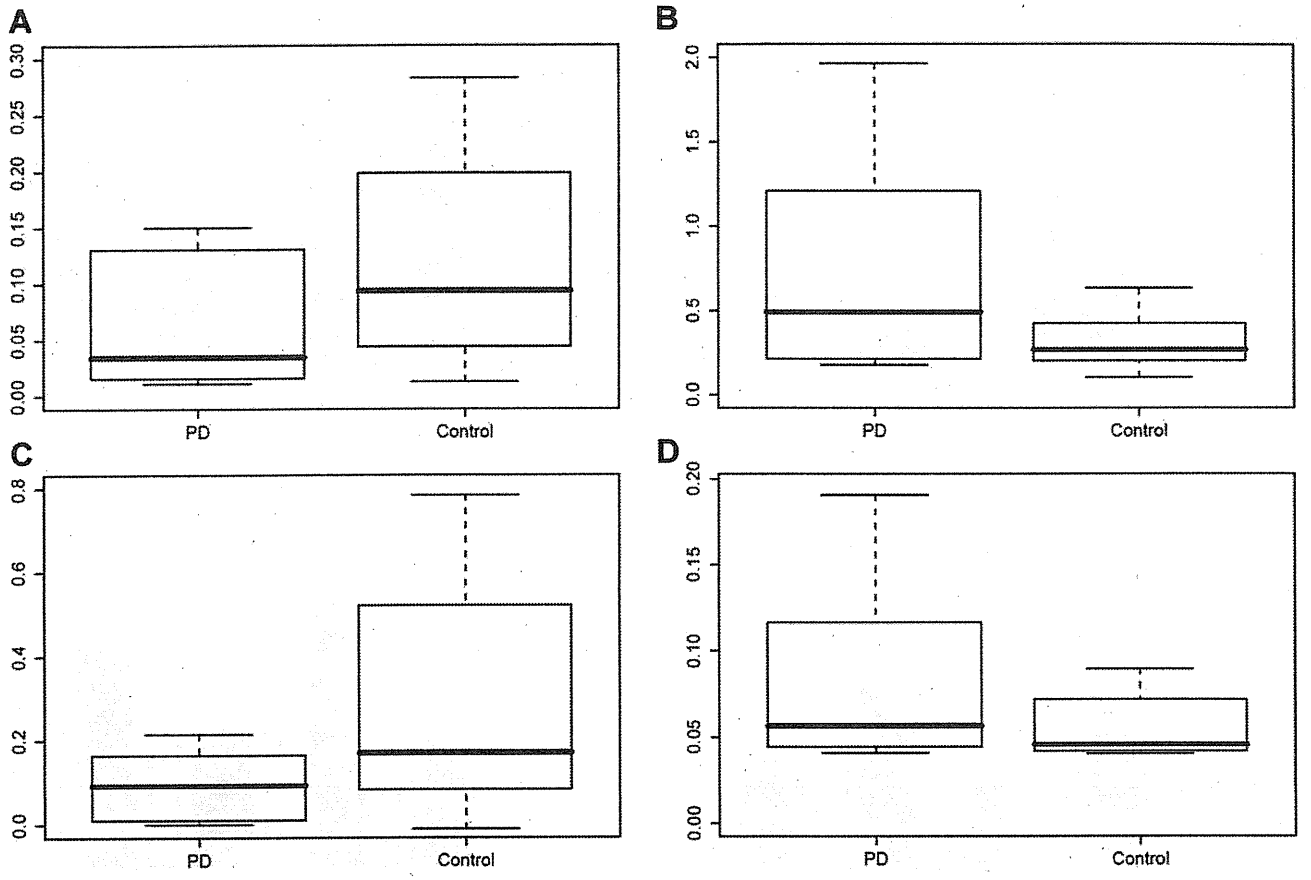

Figure 3. Box plots comparing the levels (in μg/ml) of total α-syn (*A*), pS-α-syn (*B*), oligo-α-syn (*C*), and oligo-pS-α-syn (*D*) in patients with PD compared to healthy controls. In each plot, the box extends from the lower to the upper quartile of the data, with the median indicated by a horizontal line within the box. The difference between the lower and upper quartiles is called the interquartile range (IQR). The upper and lower whiskers extend to the most extreme data values that are no more than 1.5 IQR greater than the upper quartile, and no more than 1.5 IQR less than the lower quartile, respectively.

particular sample set, this assay is less likely to have any practical value as a discriminatory diagnostic tool.

Immunoblot analysis of phosphorylated α-syn in plasma

To better characterize the phosphorylated α-syn detected in plasma, we extracted α-syn from individual PD plasma samples by immunocapture on magnetic Dynabeads and then analyzed the extracted proteins by immunoblotting. The beads were derivatized with the phosphorylation-independent α-syn antibody N-19 (Santa Cruz Biotechnology), which is the antibody used for capture in the pS-α-syn immunoassay. Proteins eluted from the beads were detected by immunoblotting with two different α-syn antibodies: phosphorylation-dependent rabbit monoclonal antibody, pS129, which is the detection antibody used in the pS-α-syn immunoassay (**Fig. 5*A***), and the phosphorylation-independent rabbit polyclonal antibody, FL-140 (Fig. 5*B*). Rabbit anti-ubiquitin antibody FL-76 (Santa Cruz Biotechnology) was used to determine whether any of the bands represented ubiquitinated forms of α-syn (Fig. 5*C*).

The plasma samples were chosen according to the immunoassay results, with one sample giving a low signal for pS-α-syn (Fig. 5, lane 2) and the other a high signal (Fig. 5, lane 3). Immunoblots using the phospho-α-syn-dependent antibody revealed immunoreactive bands from both of these plasma samples, together with the human recombinant phospho-α-syn (at ~17 kDa), but not the nonphosphorylated recombinant protein (Fig. 5*A*, lane 4). The sample derived from the high-reading plasma revealed more intense bands than the low-reading sample, at ~21, 24, and 50–60 kDa. FL-140 revealed both the phosphorylated and nonphosphorylated recombinant protein standards, and also a 24-kDa band in both plasma samples (Fig. 5*B*). The 21-kDa band detected by pS129 was absent, but an additional higher-molecular-weight smear, at >35 kDa, was present. On the basis of the size of these α-syn species, we hypothesize that the 24-kDa band may correspond to phosphorylated, monoubiquitinated α-syn. The anti-ubiquitin antibody, FL-76, strongly labeled the 24-kDa band, as well as the broad "smears" at higher molecular mass, suggesting that all of these bands represent ubiquitinated forms of α-syn (Fig. 5*C*). Control samples of 100 ng human IgG and albumin, the N-19 immunocapture antibody, and a control immunoprecipitation

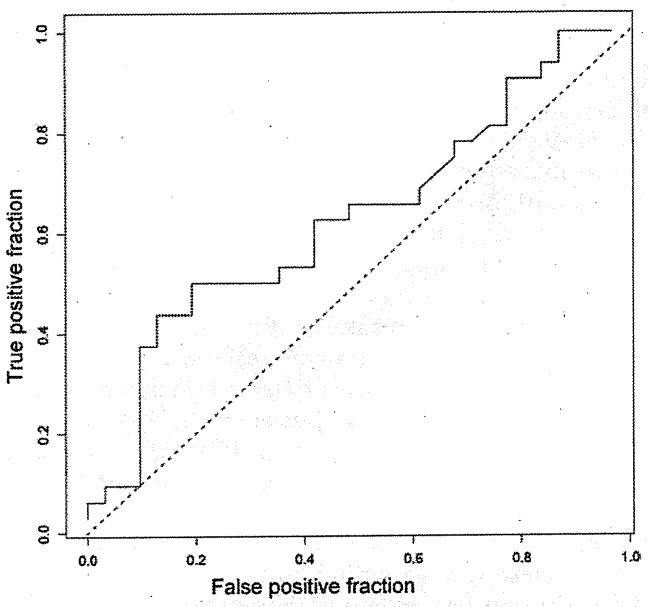

Figure 4. ROC curve showing the ability of the pS-α-syn levels to discriminate between patients with PD and healthy controls (AUC=0.68).

using PBS rather than plasma, gave no immunoreactive bands (data not shown).

DISCUSSION

α-Syn has been linked directly to the etiology of the α-synucleinopathies by mutations in and multiplication of its gene (*SNCA*) that result in familial forms of either PD or DLB. We have reported previously that α-syn is released from cells and is present in human body fluids, including CSF and blood plasma (26). This has led to considerable interest in α-syn in these body fluids as a potential biomarker for the α-synucleinopathies (30–37, 42–44). However, most of these studies have relied on immunoassays that cannot distinguish between monomeric/oligomeric and nonphosphorylated/phosphorylated forms of the protein, apart from two previous studies of oligomeric α-syn in human CSF or blood plasma (37, 44). Here, we have set up individual sandwich immunoassays that can distinguish between total α-syn (MAb 211 capture/ FL-140 detect); oligo-α-syn (Mab 211 capture and detect); pS-α-syn (N19 capture/pS129 detect); and oligo-pS-α-syn (pS129 capture and detect). Our assays for oligomeric forms of α-syn use the double-antibody approach, where the same monoclonal antibody is used for both antigen capture and detection (37, 41). This type of assay cannot detect monomers because the capture antibody occupies the only antibody-binding site available, but it can detect oligomers, because they have multiple binding sites. Our assays for phosphorylated α-syn rely on the specificity of the monoclonal antibody pS129 to α-syn phosphorylated at Ser-129 (45). As anticipated, the recombinant nonphosphorylated α-syn gave no signal in these assays. Although the absorbance/fluorescence values for each of the 4 assays were converted to protein concentrations using the relevant standard curve, due to the nature of these assays, this may only represent an estimate of concentration for that particular assay since the precise nature of the α-syn species detected in plasma has not been not determined for each assay and the native species are likely to differ from the standards prepared from the recombinant

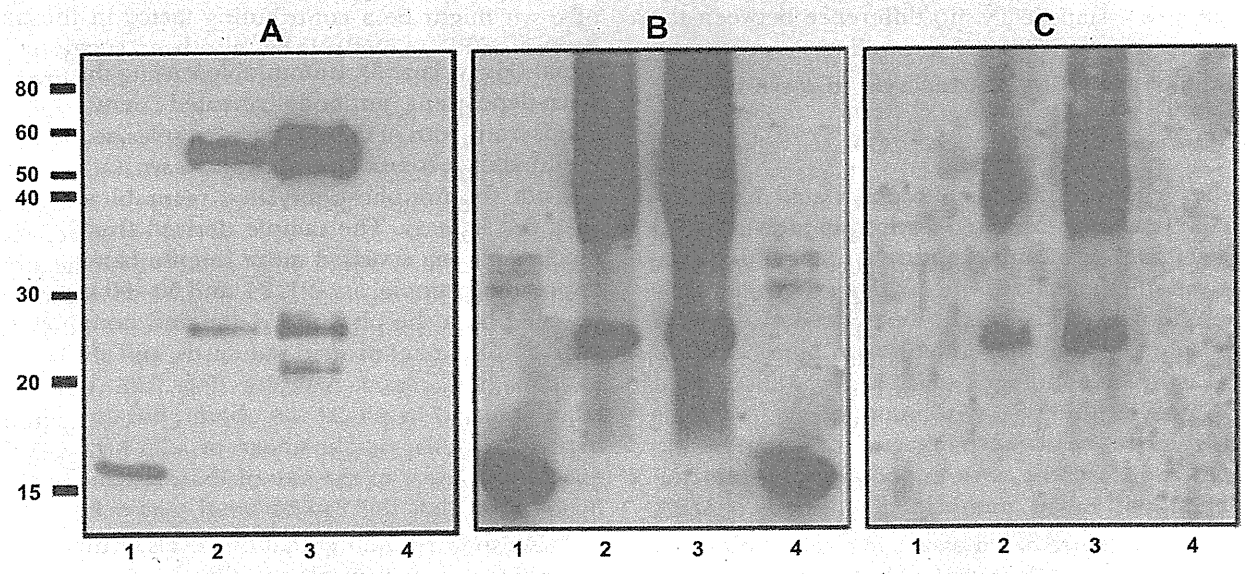

Figure 5. Immunoblot analysis of phosphorylated α-syn from plasma samples. Proteins immunocaptured from one plasma sample giving a low immunoassay signal for pS-α-syn protein (lane 2), and the other a high signal (lane 3), were immunoblotted along with recombinant phosphorylated α-syn (lane 1), and the recombinant nonphosphorylated standard (lane 4). *A*) Analysis with the phospho-dependent α-syn rabbit monoclonal antibody pS129 (Epitomics). *B*) Analysis with the rabbit polyclonal α-syn antibody FL-140 (Santa Cruz Biotechnology). Analysis with the rabbit polyclonal antiubiquitin antibody FL-76 (Santa Cruz Biotechnology).

protein. Nonetheless, the data can be compared within each individual immunoassay, although not necessarily across assays.

As far as we are aware, this is the first report to look at α-syn levels in repeat blood samples taken from individual patients with PD (collected here over an initial 3-mo period, from the first 32 patients enrolled into an ongoing longitudinal study) and also the first to detect phosphorylated forms of α-syn in blood plasma. One of the most important and novel results arising from this study is that although it is clear that the concentrations of α-syn vary greatly between individuals (for reasons that are, as yet, unknown), they remain remarkably consistent (at least over 3 mo) within the vast majority of individuals. This was a general finding for all four of the immunoassays. This lack of fluctuation of α-syn levels within individuals is a prerequisite for establishment of any viable biomarker. It has been reported that only a very small proportion of the α-syn in whole blood is present in peripheral blood mononuclear cells, platelets, and plasma, with the majority being present in red blood cells (46). Considering the abundance and fragility of red blood cells, α-syn levels in plasma, or other bodily fluids, such as CSF, could be artificially elevated in some samples by contamination with intact or lysed red blood cells (32). However, this type of contamination cannot be a confounding factor here, given the very high degree of consistency of α-syn concentrations within the plasma samples prepared from blood taken on 4 different occasions from the same individual. Moreover, it is unlikely that conditions, such as anemia would confound the results because all recruits with PD had undergone regularly blood screening for hemoglobin levels, and if anemia had been detected, it would have been treated.

The results of the 4 different immunoassays reveal that there was, statistically, no difference between the levels of total α-syn, oligo-α-syn, or oligo-pS-α-syn when comparing the 32 patients with PD with the healthy (nondiseased) control group of 30 individuals. This is not consistent with our previous findings for oligo-α-syn (37), which was elevated in PD. However, in our previous study, the control samples were obtained from individuals with serious medical conditions, such as stroke, heart disease, and cancer, and they were also taken at a different institution from the PD samples. In our current study, the controls were from healthy people, and variables such as the collection, separation and storage of the blood samples were more stringently controlled for. It should be noted that the oligo-α-syn immunoassay is the same as that reported in our previous publication (37), except for the detection system, which was changed from alkaline phosphatase (absorbance-based assay) to streptavidin-europium (time-resolved fluorescence). This has improved assay sensitivity and has allowed us to dilute the plasma samples to 1:25, whereas previously (37), the samples were not diluted. It is possible that this has also contributed to the different findings reported here. We now find that only the levels of pS-α-syn were higher in the PD group than in the control group (P=0.053), suggesting that the total phosphorylated protein may be the more useful diagnostic marker in plasma, and this is reflected in the ROC analysis for pS-α-syn (AUC=0.68). It should be noted that we did not make any formal adjustment for multiple testing (Bonferroni correction), because this is only a small-scale study, and this question will be addressed more fully in later work, when we have acquired and analyzed data from many more subjects.

The immunoblot results confirm that immunoreactive protein bands, with an intensity compatible with the immunoassay results, are detected when the protein from plasma is immunocaptured with N-19 and detected on immunoblots with pS129 (*i.e.*, the same capture and detection antibodies as those used for the pS-α-syn immunoassay). Some of these bands seem to represent ubiquitinated forms of the protein, since they also reacted with an antiubiquitin antibody. Whether these phosphorylated and ubiquitinated forms of α-syn originate from a cellular component in the blood itself or whether they originate from a peripheral tissue source elsewhere in the body, or from the brain (*via* CSF) is currently unknown.

Phosphorylation and ubiquitination are important secondary modifications of α-syn, with the protein deposited in LBs being predominantly phosphorylated at serine 129 (3, 47). Ubiquitination is a means of targeting a protein for destruction *via* the proteasome, and a defect in the ubiquitin-proteasome system is likely to be fundamental to the molecular pathogenesis of PD (48), although, recently, it has been suggested that α-syn phosphorylated at Ser-129 is targeted to the proteasome in a ubiquitin-independent manner (49). Phosphorylation of α-syn has been found to promote fibril formation, suggesting that hyperphosphorylation of α-syn might be a contributing factor in the pathogenesis of PD (47, 50). It has also been suggested that α-syn phophorylated at serine 129 and its aggregation are involved in pathway responsible for α-syn toxicity in oligodendrocytes (51, 52). Given this pathological role for phosphorylated and ubiquitinated forms of α-syn, the levels of these modified proteins in body fluids, including blood plasma, are more likely to reflect the fundamental neuropathology of PD than the normal protein (33). This inference is borne out by the results of the present study. Observations that 10–37% of aged, neurologically healthy controls display some α-syn pathology in their brains (53, 54), with about half of such subjects showing abundant α-syn pathology (53), could explain why the levels of pS-α-syn did not better discriminate between patients with PD and healthy controls.

It is also worth noting that the levels of the nonphosphorylated protein in plasma showed a weak but positive correlation with sampling age (of the patients with PD) in the present study, whereas the phosphorylated protein showed no such correlation. Age has already been noted as a confounding variable for total α-syn levels in CSF (32). This lack of correlation between

phosphorylated α-syn and age could be an additional advantage in its development as a potential molecular biomarker.

In summary, we have validated some novel assays for assessing α-syn levels in blood plasma; shown that these levels are highly consistent in repeat blood samples taken over 3 mo from patients with PD; presented evidence for the presence of pS-α-syn (phosphorylated at Ser-129) in blood plasma; and found that the mean level of pS-α-syn was marginally significantly higher (P=0.053) in the PD samples than in the controls. We accept that the latter result is preliminary and will need to be confirmed in larger-scale studies. Nevertheless, on the basis of the data presented here, further study of phosphorylated α-syn as a potential biomarker for PD and related α-synucleinopathies is clearly warranted. Moreover, whether any of the different forms of α-syn can be used to monitor the progression of PD cannot be determined from the present study with longitudinal sampling over 3 mo only and must await data from our ongoing longer-term longitudinal studies. FJ

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Neurobiology of Disease

Regulation of Mitochondrial Transport and Inter-Microtubule Spacing by Tau Phosphorylation at the Sites Hyperphosphorylated in Alzheimer's Disease

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The microtubule-associated protein Tau is a major component of the neurofibrillary tangles that serve as a neuropathological hallmark of Alzheimer's disease. Tau is a substrate for protein phosphorylation at multiple sites and occurs in tangles in a hyperphosphorylated state. However, the physiological functions of Tau phosphorylation or how it may contribute mechanistically to Alzheimer's pathophysiology are not completely understood. Here, we examined the function of human Tau phosphorylation at three sites, Ser199, Ser202, and Thr205, which together comprise the AT8 sites that mark abnormal phosphorylation in Alzheimer's disease. Overexpression of wild-type Tau or mutated forms in which these sites had been changed to either unphosphorylatable alanines or phosphomimetic aspartates inhibited mitochondrial movement in the neurite processes of PC12 cells as well as the axons of mouse brain cortical neurons. However, the greatest effects on mitochondrial translocation were induced by phosphomimetic mutations. These mutations also caused expansion of the space between microtubules in cultured cells when membrane tension was reduced by disrupting actin filaments. Thus, Tau phosphorylation at the AT8 sites may have meaningful effects on mitochondrial movement, likely by controlling microtubule spacing. Hyperphosphorylation of the AT8 sites may contribute to axonal degeneration by disrupting mitochondrial transport in Alzheimer's disease.

Introduction

Mitochondrial transport within axons is crucial for axonal maintenance, and its dysregulation can contribute to neurodegenerative diseases (Su et al., 2010). In axons, mitochondrial movement is driven by two oppositely directed motor proteins, kinesin and dynein, along microtubules (MTs) (Hollenbeck and Saxton, 2005; Bereiter-Hahn and Jendrach, 2010). The surface of MTs is decorated with microtubule-associated proteins (MAPs) (Marx et al., 2006; Vershinin et al., 2007). Tau serves as a predominant MAP in axons and is a filamentous protein of 441 amino acid residues (the longest human isoform). Tau is comprised of two functional regions, the N-terminal projection domain that protrudes from the surface of MTs and the C-terminal MT-binding domain. Overexpression of Tau inhibits mitochondrial transport in various cells (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002; Dixit et al., 2008; Stoothoff et al., 2009; Vossel et al., 2010). However, the mechanism and regulation of Tau-mediated inhibition of mitochondrial transport are not understood.

Tau is a major component of neurofibrillary tangles found in Alzheimer pathology. Tau is also a phosphoprotein, the functions of which can be regulated by phosphorylation (Stoothoff and Johnson, 2005; Hanger et al., 2009). Many of the phosphorylation sites reside in proline-directed (Ser/Thr)-Pro sequences. These sites are moderately phosphorylated in healthy neurons. However, hyperphosphorylation is linked to neurodegeneration with phosphorylation of more than 20 sites shown in the degenerated brains of Alzheimer's patients (Watanabe et al., 1993; Morishima-Kawashima et al., 1995; Stoothoff and Johnson, 2005). Cdk5 and GSK3β are two proline-directed protein kinases that are known to phosphorylate these (Ser/Thr)-Pro sites (Ishiguro et al., 1992; Planel et al., 2002). Furthermore, hyperactivation of Cdk5 or GSK3β reduces mitochondrial movement (Darios et al., 2005; Morel et al., 2010). However, it has not been clearly demonstrated whether Tau is a major downstream target of these kinases and, if so, which phosphorylation site(s) is critical. Among GSK3β- and Cdk5-related phosphorylation sites, Ser199, Ser202, and Thr205 are particu-

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Epitope mapping of antibodies against TDP-43 and detection of protease-resistant fragments of pathological TDP-43 in amyotrophic lateral sclerosis and frontotemporal lobar degeneration

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ABSTRACT

TAR DNA-binding protein of 43 kDa (TDP-43) is the major component of the intracellular inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Here, we show that both monoclonal (60019-2-Ig) and polyclonal (10782-2-AP) anti-TDP-43 antibodies recognize amino acids 203–209 of human TDP-43. The monoclonal antibody labeled human TDP-43 by recognizing Glu204, Asp205 and Arg208, but failed to react with mouse TDP-43. The antibodies stained the abnormally phosphorylated C-terminal fragments of 24–26 kDa in addition to normal TDP-43 in ALS and FTLD brains. Immunoblot analysis after protease treatment demonstrated that the epitope of the antibodies (residues 203–209) constitutes part of the protease-resistant domain of TDP-43 aggregates which determine a common characteristic of the pathological TDP-43 in both ALS and FTLD-TDP. The antibodies and methods used in this study will be useful for the characterization of abnormal TDP-43 in human materials, as well as in vitro and animal models for TDP-43 proteinopathies.

1. Introduction

TDP-43 is a nuclear ribonucleoprotein implicated in exon splicing, gene transcription, regulation of mRNA stability, mRNA biosynthesis, and formation of nuclear bodies [1–5]. It has been identified as the major component of the ubiquitin-positive tau-negative intracytoplasmic inclusions in frontotemporal lobar degeneration (FTLD), amyotrophic lateral sclerosis (ALS) [6,7] and other neurodegenerative disorders [8–12]. Identification of mutations in familial and sporadic ALS and FTLD cases demonstrated a direct link between the genetic lesion and development of TDP-43 pathology [13–16]. Immunohistochemical studies using anti-TDP-43 antibodies revealed that TDP-43 translocates from its normal nuclear localization into the cytoplasm in these disorders. Furthermore, biochemical analysis detected abnormally phosphorylated TDP-43 of 45 kDa, high-molecular-weight smearing and C-terminal fragments of approximately 25 kDa, as well as normal TDP-43 of 43 kDa in the detergent-insoluble, urea-soluble fraction from affected brains. The antibodies generated by immunizing C-terminal phosphopeptides of TDP-43, such as pS409/410 and pS403/404, strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulations in neuropathological examination [17]. Immunoblotting of the Sarkosyl-insoluble fractions from FTLD and ALS cases using these phosphospecific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 of 45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD and ALS, and the band patterns of the C-terminal fragments of phosphorylated TDP-43 correspond to the neuropathological subtypes.

Anti-TDP-43 monoclonal antibody (mAb) (60019-2-Ig; Proteintech Group Inc., Chicago, IL) and polyclonal antibody (pAb) (10782-2-AP; Proteintech Group Inc., Chicago, IL) are widely used for the investigation of TDP-43 pathology [6,7,9,18–21]. According to the manufacturer's specifications, anti-TDP-43 mAb and pAb were generated against the N-terminal 260 amino acids (aa) of the protein, but the precise epitope has not yet been identified. Another mouse monoclonal antibody against TDP-43 (2E2-D3; Abnova Corporation, Taipei, Taiwan) is also commercially available; it recognizes residues 205–222 of human TDP-43, but does not recognize mouse or rat TDP-43 [22].

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