PHOSPHORYLATION OF SER 129 IS THE DOMINANT PATHOLOGICAL MODIFICATION OF α-SYNUCLEIN IN FAMILIAL AND SPORADIC LEWY BODY DISEASE

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Running Title: α-Synuclein in Lewy bodies

A comprehensive, unbiased inventory of synuclein forms present in Lewy bodies from patients with dementia with Lewy bodies was carried out using 2D immunoblot analysis, novel sandwich ELISAs with modification-specific synuclein antibodies, and mass spectroscopy. The predominant modification of α–synuclein in Lewy bodies is a single phosphorylation at Ser 129. In addition, there is a set of characteristic modifications that are present to a lesser extent, including ubiquitination at Lys residues 12, 21 and 23 and specific truncations at Asp 115, Asp 119, Asn 122, Tyr 133 and Asp 135. No other modifications are detectable by MS/MS mapping, except for a ubiquitous N-terminal acetylation. Small amounts of phosphorylated Ser 129 and Asp 119-truncated α–synuclein are present in the soluble fraction of both normal and disease brains, suggesting that these Lewy body-associated forms are produced during normal metabolism of α–synuclein. In contrast, ubiquitination is only detected in Lewy bodies and is primarily present on phosphorylated synuclein; it therefore likely occurs after phosphorylated synuclein has deposited into Lewy bodies. This invariant pattern of specific phosphorylation, truncation and ubiquitination is also present in the detergent-insoluble fraction of brain from patients with familial Parkinson’s disease (synuclein A53T mutation) as well as multiple system atrophy, suggesting a common pathogenic pathway for both genetic and sporadic Lewy body diseases. These observations are most consistent with a model in which preferential accumulation of normally produced Ser 129 phosphorylated α–synuclein is the key event responsible for the formation of Lewy bodies in various Lewy body diseases.

A number of neurodegenerative diseases including Parkinson’s disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) are defined histologically by the presence of Lewy bodies (LBs), intracellular protein aggregates that have a range of morphologies - from cytoplasmic spheres to neuritic threads also referred to as Lewy neurites (LNs). A number of proteins have been identified in LBs largely by immunohistochemical staining of brain, although the two most common are ubiquitin and α-synuclein (1-4). The invariable presence of α-synuclein in LBs suggests that it plays a key role in the etiology of such diseases (“synucleinopathies”). Point mutations in the synuclein gene as well as multiplication of the gene in familial cases of PD lead to autosomally dominant familial forms of PD (5-9). As in sporadic PD, LBs are also found in the brains of individuals with familial PD suggesting that clues about synuclein’s pathogenic role lie within the LB.

Since α-synuclein is a relatively abundant neuronal protein, and LBs are found in diseased brain, we hypothesized that the formation of the abnormal LB structures results from specific modifications to this protein. We therefore analyzed the specific forms of α-synuclein that are found in LBs isolated from patients with DLB using a combination of 2D immunoblots, immunoaffinity purification and LC-MS/MS. We
found that a single phosphorylation at Ser 129 (10) of otherwise unmodified α-synuclein is the most abundant modification of the synuclein in LBs. Smaller amounts of ubiquitinated phosphorylated synuclein, as well as synuclein truncated at Asp 119 were also present in every case. In addition, novel truncations at Asp 115, Tyr 133 and Asp 135 were detected. LB-enriched fractions from a member of the Contursi kindred, a family with an A53T mutation in α-synuclein that co-segregates with an autosomally dominant familial form of PD, and glial cell inclusions (GCIs) from an MSA patient had the same profile of modifications as did the DLB cases, thus suggesting a common pathway to pathogenesis in both sporadic and familial synucleinopathies. Small amounts of phosphorylated Ser 129 (P-S129) synuclein were found in the soluble fraction of both control and disease brain. This normally produced, cytosolic form of α-synuclein may thus be the precursor to the predominant pathogenic form of synuclein in LBs. Synuclein truncated at Asp 119 is also found outside of the LBs, in both normal and disease brain, suggesting that this is a normal metabolite of α-synuclein. In contrast, ubiquitinated synuclein, predominantly phosphorylated at Ser 129, was only found in LBs, and therefore most likely occurs after deposition of P-S129 synuclein into LBs.

EXPERIMENTAL PROCEDURES

Recombinant α-synuclein - Clones of full-length and C-terminally truncated (terminating at amino acid 119 or 122) human α-synuclein in pET21d vectors were transformed into the BL21(DE3) strain of E. coli. All clones were verified by sequencing. Synuclein expression was induced with 1 mM IPTG, and bacteria were harvested 2 hours post-induction by spinning at 6500g for 15 minutes. The E coli pellet was lysed by sonication for 3.5 minutes in 50 mM Tris, pH 7.5, 0.1 mM DTT and Complete Protease Inhibitor Cocktail (Roche Diagnostics). Cellular debris was pelleted by spinning at 38,000g for 20 min. The resulting supernate was supplemented with an additional 10 mM DTT and then placed in a boiling water bath for 10 minutes. Samples were centrifuged as above and the supernate containing the α-synuclein was applied to a HiTrap Q-Sepharose column (Amersham Biosciences) equilibrated in lysis buffer. Synuclein was eluted with a linear NaCl gradient (10 to 700 mM). α-Synuclein preparations were >95% pure by SDS-PAGE. Protein concentration was measured by the BCA assay (Pierce Biotechnology, Rockford, IL) and confirmed by amino acid analysis (AAA Laboratory, Mercer Island, WA). MS analysis of the purified samples established that the full-length synuclein was intact and that the truncated synuclein terminated with either Asp 119 or Asn 122.

In vitro phosphorylation of α-synuclein at Ser 129 - Recombinant α-synuclein (1 mg/ml) was incubated overnight at 30°C with 3 mU/µl of casein kinase 2 (Upstate) in 20 mM Tris, pH 8, 4 mM MgCl₂, 130 mM KCl, 5 ng/µl poly-L-Lys (Sigma) and 800 µM ATP (Sigma). Phosphorylation of Ser 129 was confirmed by LC-MS/MS and the phosphorylated Ser 129 specific antibody 11A5. Phosphorylation at other sites was not detected by LC-MS/MS.

Antibodies - Monoclonal antibodies 1H7 and 9E4 were generated in mice injected with purified recombinant α-synuclein expressed in and purified from E coli. α-Synuclein purified from bovine brain according to a modified procedure of Jakes et al. (11) was used as the antigen for production of the mouse monoclonal antibodies 5C12 and 8A5. The antigen for the P-S129 specific antibody 11A5 was the peptide CAYEMPSEEGYQ with a phosphorylated Ser. This peptide corresponds to amino acids 124-134 of α-synuclein with a Cys residue added at the N-terminus for coupling and is identical to that described by Fujiwara et al (10). A clonal cell line producing the phospho-specific antibody was obtained by counter-screening against the non-phosphorylated peptide. The rabbit polyclonal antibodies EL43, EL47 and EL48 were prepared to synthetic peptides (AnaSpec; San Jose, CA) corresponding respectively to amino acids 1-12, 115-122, and 131-140 of α-synuclein with a GGC linker on the C-terminus of each peptide. The neo-epitope polyclonal antibody EL101 was generated by immunizing rabbits with peptides corresponding to amino acids 115-119 (DMPVD) with a CGG linker on the N-terminus of each peptide. All peptides for polyclonal antibody production were coupled through the Cys residue to Imject 2
SuperCarrier Immune Modulator (Pierce) while peptides used for monoclonal antibody production were prepared by coupling the Cys on the peptide to sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories; West Grove, PA) using sulfo-EMCS (Molecular Biosciences; Boulder CO). Monoclonal antibodies were purified by anion exchange chromatography or on a Protein A Sepharose column, and rabbit polyclonal antibodies were affinity purified on NHS-activated Sepharose (Pharmacia Biotech) conjugated to the appropriate peptide antigen. Antibodies were epitope mapped as described below and tested for specificity to α- versus β-synuclein using recombinant protein.

Commercial antibodies used for immunoblot analysis include Syn-1 (BD Transduction Laboratories), an anti-ubiquitin antibody (Z0458; DAKO), and the HRP-labeled secondary antibodies, donkey anti-rabbit and goat anti-mouse (Amersham Biosciences).

**Epitope mapping of synuclein antibodies** - Monoclonal and polyclonal antibodies generated against α-synuclein and the monoclonal antibody Syn-1 were epitope mapped using a series of overlapping, linear peptides covering the full-length of the α-synuclein sequence (Mimotopes, Melbourne, Australia). The 15 amino acid-long peptides overlapped by 12 amino acids with 3 amino acid steps. All peptides were biotinylated at the C-terminus; however, the last 3 peptides in the series were repeated with biotinylation at the N-terminus to allow for the detection of C-terminal specific antibodies. Peptides (5 nM) were captured on streptavidin-coated ELISA plates (Pierce) which were washed and then incubated with purified antibody (2 µg/ml) for 1 hour. Antibody binding to peptide was detected by a colorimetric ELISA assay using HRP-conjugated anti-mouse antibody. Epitopes were mapped with a resolution of two amino acid residues, determined by the three residue increments in sequence coverage by the mapping peptides. The polyclonals EL43 and EL48 were mapped respectively to amino acids 1-3 and 118-123 of α-synuclein. The monoclonal 1H7 was mapped to amino acids 91-99. Other antibodies used in this study and their epitope mapped sites are cited in Table 1.

**Synuclein ELISA assays** - Total and P-S129 α-synuclein levels were quantitated by sandwich ELISAs utilizing 1H7 as the capture antibody and either 5C12 or 11A5 as a reporter antibody, respectively. All steps were carried out at room temperature. Both ELISAs were performed on 4HBX ultra high binding plates (Immulon) coated overnight at 4°C with 10 μg/ml of 1H7. Plates were blocked for 1 hr with Casein Blocking Solution (0.25% casein in phosphate buffered saline, 0.05% sodium azide). Samples to be assayed were extracted for 1 hour with 5 M guanidine plus Complete Protease Inhibitor Cocktail (Roche Diagnostics). Both samples and standards (recombinant α-synuclein +/- in vitro phosphorylation) were diluted to 0.5 M guanidine in Casein Blocking Solution, and were applied in triplicate in 100 µl aliquots to the coated, blocked plates. Following an overnight incubation at 4°C, plates were brought to room temperature, washed 4 times with 400 µl of TBST (150 mM sodium chloride, 50 mM Tris, pH 7.5, 0.1% Tween-20) per wash and then incubated for 1 hour at room temperature with biotinylated 5C12 or 11A5 (2 µg/ml). After washing, plates were incubated for 1 hr with HRP-labeled avidin D (Vector Laboratories, Burlingame, CA) or avidin (Amersham Biosciences) diluted 1:7500 and 1:10,000, respectively in Specimen Diluent (0.6% globulin-free BSA, 1.5 mM monobasic sodium phosphate, 8 mM dibasic sodium phosphate, 145 mM sodium chloride, 0.05% Triton X-405 with 0.05% thimerosal). Plates were washed and incubated with TMB1 Component HRP Microwell Substrate (BioFX Laboratories, Ownings Mills, MD) for 5 minutes to initiate the colorimetric reaction which was stopped with 450 mM STOP Reagent (BioFX Laboratories). Plates were read at 450 nm.

**Isolation of LBs and GCIs** - Individuals with DLB and MSA as well as control patients whose brains were used in this study are described in Suppl. Table 1. LBs, together with LN, were isolated as described by Jensen et al (12) from the cingulate, temporal and frontal grey matter of individuals diagnosed with DLB. A mock LB preparation was performed with human brain tissue from control individuals (91/290 and 91/204). GCIs were prepared from an MSA brain (P28) as described by Gai et al (13). All preparations were

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solubilized by two sequential extractions with IEF lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) for 1 to 3 hrs at room temperature and 10 minutes of bath sonication.

**Preparation of soluble and particulate human brain fractions** - Cortical grey matter was homogenized in 4 volumes of 0.32 M sucrose, 50 mM Tris, pH 7.5, 2 M thiourea, 4% CHAPS) for 1 to 3 hrs at room temperature and 10 minutes of bath sonication. The resulting post-nuclear supernate was centrifuged at 100,000g for 30 min producing a soluble and particulate fraction. The particulate fraction was solubilized in IEF lysis buffer or in Laemmli sample buffer for analysis by 2D or 1D PAGE, respectively.

**Synuclein purification from human brain** - α-Synuclein was purified from 35 g of hippocampal, temporal, cingulate and prefrontal cortical grey matter combined from individuals (patients AD22 and P52; Suppl. Table 1) with DLB. Brain material was homogenized in 4 volumes of sucrose buffer (50 mM Tris, pH 7.5, 50 mM sucrose, 5 mM EDTA, 5 mM DTT) plus Complete Protease Inhibitor Cocktail (Roche Diagnostics) and centrifuged at 100,000g to sediment LBs. The pellet was extracted in 200 ml of UTC (7 M urea, 2 M thiourea, 4% CHAPS) for 2 hrs at 25°C. The resulting extract was clarified by centrifugation and filtration and then applied to a 16/10 Q-Sepharose column (Amersham) equilibrated in anion-exchange buffer (50 mM Tris, pH 7.5, 5 M urea, 5 mM EDTA, 5 mM DTT). Twenty column volumes of anion-exchange buffer with a 10 to 700 mM NaCl gradient was applied, and α-synuclein was found to elute between 200-300 mM NaCl. Fractions were pooled on western blots for α-synuclein immunoreactivity, and were pooled into aliquots enriched in either truncated α-synuclein (pools 1 and 2/3) or P-S129 α-synuclein (pool 4). Pools were diluted with sucrose buffer to a final concentration of 0.5 M urea and further purified by immunoaffinity chromatography using the α-synuclein specific monoclonal antibody 5C12 or the P-S129 specific antibody 11A5 conjugated to NHS-Activated Sepharose 4 Fast Flow (3-5 mg antibody/ml resin). The resin was washed with 50 mM Tris, pH 7.5, 100 mM NaCl, and α-synuclein was eluted with 200 mM glycine, pH 2.8, 500 mM NaCl.

**Extraction of Contursi brain** - A temporal lobe cortex specimen from the brain of a 57-year old woman with a heterozygous A53T α-synuclein mutation (Contursi kindred member IX/56; 14) was obtained from Drs. D. Dickson and L. Golbe. A specimen of control brain from a 59-year old female who died of chronic obstructive pulmonary disease (postmortem interval, 15 hrs) was obtained from Dr. Jennifer A. Chan. Temporal cortex specimens were homogenized in parallel in 3 volumes (ml/g) of 50 mM Tris, pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.2% NP-40 and Complete Protease Inhibitor Cocktail (Roche Diagnostics), as described by Schlossmacher and Shimura (15) and then centrifuged at 100,000g for 30 min at 4°C. The pellet was extracted with the homogenization buffer containing 1% Triton X-100 substituted in place of NP40, extracted for 2 hours with intermittent vortexing, and spun as above. The pellet was then homogenized in 20 mM Tris, pH 7.5, 0.32 M sucrose, 1 mM magnesium sulfate with complete protease inhibitors, and centrifuged at 500g for 15 min at 4°C to generate a supernate (“TBS extract”) and pellet. The pellet was sequentially extracted with 4 volumes of 50 mM Tris, pH 7.5, 8 M urea, 1 mM EDTA and 1 mM EGTA plus protease inhibitors (“Urea extract”) followed by the same buffer containing 1% SDS (“SDS extract”). Extracts were spun at 100,000g for 30 min.

**PAGE and Immunoblotting** – For 2D analysis, 50-100 µg of LB protein or 100-200 µg of protein from the soluble fraction of brain were focused on 24 cm Immobiline DryStrip gels (Amersham Biosciences) with either a pH 4-7 or pH 3-10 gradient. Strips were run on DALT gels (25 X 21 cm, 12.5% acrylamide). 1D analysis was performed using tricine buffered 10-20% acrylamide gels (Invitrogen, Carlsbad, CA). 1D gels were blotted onto PVDF (Millipore) or nitrocellulose (Invitrogen) membranes at 1 amp for 1.5 hours using Laemmli transfer buffer. 2D gels and some 1D gels were blotted onto PVDF for one hour 15 minutes at 0.8 V/cm² using a Multiphor semi-dry blotting apparatus with a discontinuous buffer system as specified by the manufacturer. All membranes were placed in boiling PBS for 3 minutes prior to blocking with
either 5% milk or 10% newborn calf sera (Sigma) in Tris-buffered saline (50 mM Tris, pH 7.5, 150 mM sodium chloride). Immunoreactivity was detected using ECL or ECL Plus (Amersham Biosciences). The apparent molecular weights of synuclein species were extrapolated from the migration of either BioRad Precision or Amersham Rainbow molecular weight markers on 1D gels. For reprobing a blot with additional antibodies, the blot was stripped by incubation in 5 M guanidine hydrochloride for at least 30 minutes, followed by two 5 minute washes in Tris-buffered saline and blocking. Complete stripping was verified by reprobing with the appropriate secondary antibody. The pI values noted on each blot were estimated from calibration curves supplied by the manufacturer of the isoelectric focusing strips.

Nanospray-ESI-MS/MS Analysis - Plugs were removed from 1D SDS PAGE gels using an Ettan Spotpicker (GE Healthcare/Amersham Biosciences), then equilibrated in 25 mM ammonium bicarbonate and 50% acetonitrile, dehydrated in acetonitrile, and rehydrated in 10 ng/µL trypsin (Promega) in 25 mM ammonium bicarbonate. Samples were acidified to pH 2 with 10% trifluoroacetic acid and sonicated to extract the peptides from the gel plugs. Protein samples in solution were enzymatically digested with 1ng trypsin (Promega; Madison, WI) suspended in 25 mM ammonium bicarbonate. Digestions were limited to 1 hour at room temperature, with partial proteolysis preferred to allow overlapping peptide sequence coverage by LC-MS/MS. Nanospray HPLC separation was performed using a Michrom Magic 2002 capillary HPLC (Michrom Bioresources; Auburn, CA) equipped with a 75 µm x 5 cm Proteopep2 C18 reversed phase nanospray column (New Objective; Cambridge, MA). Peptides were eluted with a linear gradient of 5-50% acetonitrile and 0.01% TFA in 45 minutes with a flow rate of 200 nL/min. Peptides were analyzed by MS/MS using an LCQ DecaXP ion trap mass spectrometer (ThermoElectron Corp; San Jose, CA) with spray voltage set at 2.1 kV and collision energy at 35%. Mass scanning ranges varied accordingly to detect peptides of interest. The mass acquisition method consisted of one full MS survey scan followed by four MS/MS scans of the most abundant precursor ions from the survey scan. Dynamic exclusion of MS/MS spectra was set to 30 seconds.

Database Searching and Filtering of Results - MS/MS spectra were searched using the Turbosequest algorithm within the Bioworks 3.2 software suite (ThermoElectron, San Jose, CA). Data files were searched using a custom database designed to detect serial C-terminal truncations of the α-synuclein protein and also with the SWISS-PROT database (Release 46) from Expasy. The mass tolerance of the intact precursor and fragment ions was set at 2 and 1 Da respectively. Therefore the intact peptide and peptide fragments were within +/- 1 and 0.5 Da respectively of that of the assigned peptides. The MS/MS data files were searched specifying cleavage by trypsin and allowing up to four missed cleavages. Results were filtered using two independent measures for the accuracy of matches: p-values and a cross correlation (XCorr) score, a value based on the number and intensity of ions matched in the database. The minimum acceptable Xcorr values for assigning peptides, 1.8 (+1), 2.5 (+2), and 3.0 (+3), are within the standard cutoffs typically used for MS database matches and were selected to screen out all but the most stringent matches. XCorr cutoff values were reduced to 2.5 for matching of (+3) phosphate containing peptides due to poor fragmentation patterns. Only peptide matches with p-values < 0.05, i.e. greater than 95% confidence, were accepted in assigning peptides. Differential modifications were specified as Met oxidation (16 Da) and acetylation (42 Da), and Ser, Thr or Tyr phosphorylation (80 Da). For determination of sites of ubiquitination, a differential modification of 114 Da was specified for Lys residues. This corresponds to the mass of the diglycine-modification left behind after tryptic digestion of the ubiquitinated protein.

Deconvolution of Intact Protein ESI-MS Spectrum - The charge envelope generated by ESI-MS of the intact P-S129-α-synuclein was acquired over a mass range of 600-1800 m/z and deconvoluted using Bioworks 3.2 (ThermoElectron; San Jose, CA) with a specified mass range of 5000-20000.

Immunohistochemistry of human brain - Immunohistochemical analysis was conducted on brain samples from pathologically confirmed cases of DLB (n=6), age-matched controls (n=6) and individuals with an A53T α-synuclein mutation (n=2). The patients with the synuclein mutation,
II-5 and II-6, were part of an Australian family of Greek origin (16). Brains from the control and DLB patients were bisected with one half fixed by immersion in buffered 4% formaldehyde and the other half frozen at -70°C for biochemical studies (17). Free-floating sections (50 µm) were prepared from brain regions known to contain LBs (cingulate, frontal, and temporal cortices and brainstem). Sections were incubated with primary antibodies overnight, followed by biotinylated donkey anti-rabbit or anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories). Sections were incubated with an avidin-biotinylated peroxidase complex (1:200; Vectastain ABC kit; Vector) and developed with DAB tetrahydrochloride and hydrogen peroxide. Primary antibodies used included EL48 and EL101 each used at 1µg/ml. In order to test the neo-epitope specificity of EL101 on brain sections, EL101 was pre-incubated with recombinant α-synuclein (100 µg/ml) or with the peptide antigen (20 µg/ml) prior to staining.

RESULTS

α-Synuclein in LBs - In order to determine whether differences exist between normal cytosolic and pathologically deposited α-synuclein, LBs were isolated from the brains of individuals with DLB, and cytosolic fractions were prepared from both normal and DLB brains (patient data is summarized in Suppl. Table 1). Soluble fractions were resolved by 2D gels, and the α-synuclein species were detected on immunoblots with the α-synuclein specific antibody Syn-1. The patterns from control individuals and DLB patients were qualitatively indistinguishable from each other (Suppl. fig. 1), with a representative immunoblot shown in Fig 1A. Complete sequence coverage of the most abundant α-synuclein species in this soluble fraction of normal human brain (marked with a white asterisk) was obtained from a tryptic digest of a gel plug and from purified protein using LC-MS/MS. Both sets of data demonstrate that this synuclein species corresponds to N-terminally acetylated, full-length α-synuclein which is not otherwise modified (data not shown).

Synuclein species detected in LB preparations were not found in corresponding mock preparations from control brains indicating that the synuclein in the preparations was associated with pathology (Fig. 1E). Furthermore, neither β- or γ-synuclein was detected in the preparations (data not shown). The α-synuclein species in four different LB preparations were resolved on 2D gels and detected on immunoblots with the antibody Syn-1 (Suppl. fig. 2 B-E). The pattern of synuclein species was similar between LB preparations and also similar to that of glial cell inclusions (GCIs) prepared from MSA brain (Suppl. fig. 2F). A representative immunoblot from a LB preparation is shown in Fig. 1B and compared to that of soluble synuclein from a control brain. A series of α-synuclein species were unique to or appeared to be enriched in LBs (boxes, Fig. 1B): full-length species more acidic than that of unmodified synuclein (solid box), truncated synuclein species (dashed box; shown at higher magnification in Figs. 1C and D), and high molecular weight synuclein species (dotted box). These 3 classes of modified synuclein species are discussed in further detail.

Acidic synuclein species - Full-length synuclein, modified only by N-terminal acetylation, was identified as the major synuclein species in brain cytosol (Fig. 1A). However, it is not the most abundant synuclein species in LBs. A series of more acidic spots flanking both full-length and the 15 kDa truncated synuclein are of greater abundance in LBs than in soluble protein (Fig. 2 A and B). These acidic synuclein species in both LBs and soluble brain are phosphorylated at Ser 129 as demonstrated by their immunoreactivity with the P-S129 specific antibody 11A5 (Fig. 2C and D; Suppl. fig. 3). The addition of a negatively charged phosphate is consistent with the acidic shift observed on the 2D gels.

The amount of P-S129 synuclein in LBs versus the soluble fraction of brain was directly compared on immunoblots and by ELISA assays. LBs and the soluble fraction from both normal and DLB brain were probed on the same 1D immunoblot for levels of total and P-S129 α-synuclein with the antibodies Syn-1 and 11A5 respectively (Fig. 3A and B). These acidic synuclein species in both LBs and soluble brain are phosphorylated at Ser 129 as demonstrated by their immunoreactivity with the P-S129 specific antibody 11A5 (Fig. 2C and D; Suppl. fig. 3). The addition of a negatively charged phosphate is consistent with the acidic shift observed on the 2D gels.

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S129 α-synuclein demonstrated that the ratio of P-S129 to total α-synuclein in the LB preparations varied by less than 2-fold between patients (Fig. 2E) with no correlation between P-S129 levels and postmortem interval or patient age (Suppl. fig. 1). Moreover the assays confirmed that all LB preparations examined had higher levels of P-S129 synuclein than in the soluble fraction of brain, the levels of which fell below the limit of quantitation of the assay (Fig. 2E).

In order to more quantitatively assess the enrichment of P-S129 synuclein in LBs, brains from both control individuals and DLB patients were examined. The soluble and LB-enriched particulate fractions of DLB brain were probed on 1D immunoblots with Syn-1 and 11A5 (Suppl. Fig. 4A and B). Quantitation of the blots, shown in Fig. 4A and B, demonstrates an approximate 2-fold increase in the amount of synuclein in the particulate fraction of DLB brain, most likely due to synuclein deposition into LBs. The most striking difference, however, was that the level of P-S129 synuclein in this same fraction was approximately 30 times higher than in the control particulate fraction indicating a highly selective enrichment of P-S129 synuclein. The P-S129 α-synuclein in DLB particulate fractions almost completely resisted extraction in nondenaturing detergents (Fig. 5B), indicating its presence as an insoluble component of LBs. In contrast, non-phosphorylated α-synuclein in the particulate fraction of both control and DLB brain was readily extractable (Fig. 5A).

The increased level of P-S129 synuclein in LBs was observed in other synucleinopathies as well as DLB. A GCI preparation from MSA had high levels of P-S129 synuclein as seen by an increase in the amount of acidic-shifted synuclein on 2D immunoblots and by ELISA (Suppl. fig. 2F; Fig. 2E). P-S129 synuclein was previously detected in GCIs in brain sections by immunohistochemistry with a P-S129 synuclein antibody (10, 18). The Triton X-100 insoluble brain fraction from a familial form of PD, a member of the Contursi kindred bearing an A53T α-synuclein mutation, was compared to that of control brain on 1D immunoblots probed with Syn-1 and 11A5 (Fig. 6). The α-synuclein in the Contursi sample was highly insoluble compared to that in control brain, consistent with it being in LBs, and moreover it was highly phosphorylated at Ser 129.

The presence of multiple acidic, 11A5 immunoreactive spots on 2D blots of LB preparations raised the possibility that P-S129 synuclein may have undergone additional acidic modifications such as phosphorylation at other sites. To address this possibility, P-S129 α-synuclein was purified from the LB-rich fraction of DLB brain by using anion-exchange and immunoaffinity (11A5-conjugated Sepharose) chromatography. Complete sequence coverage of tryptic digests of the purified protein by LC-MS/MS identified phosphorylation at Ser 129 as the only post-translational modification besides N-terminal acetylation (Supp. Table 2). Identical data was obtained from analysis of each of the individual acidic, 11A5-immunoreactive spots picked from a 2D gel (data not shown). No additional phosphorylation sites were identified by LC-MS/MS (with complete sequence coverage) in any of the fractions of synuclein purified from DLB brain. Furthermore, the molecular mass of the phosphorylated synuclein as determined by electrospray MS was 14578 Da (Suppl. fig. 5), consistent with the addition of a single phosphate (80 Da) to N-termally acetylated synuclein (14502 Da). Other modifications that may be present (e.g. ubiquitination, see below) are at levels far below that of P-S129. Thus, synuclein in LBs is predominantly phosphorylated at one site, Ser 129, and the phosphorylation occurs as an isolated post-translational modification without evidence for any other co-incidental post-translational modification. The train of acidic, P-S129 synuclein spots on 2D gels therefore appears to result from the “stuttering” of P-S129 synuclein during focusing in the first-dimension.

Truncated synuclein - In addition to the full-length, 16 kDa α-synuclein, a series of truncated species with apparent molecular masses of 10-15 kDa was present in LBs (Fig 1B, D, and E). All of the truncated species reacted with an N-terminal synuclein antibody but did not react with the C-terminal antibody 8A5 (Table 1), indicating that they are C-terminally truncated. The most striking difference between the truncations in the LBs versus that in the cytosol was the presence of the 11.5 and 13.5 kDa species only in LB preparations (Fig.1). Although the 10 kDa truncation appears to be unique to LBs in Fig. 1, it was detectable at
higher loadings of soluble protein (data not shown).

In order to be able to assess whether the individual truncated species were enriched in LBs or altered in the cytosol of diseased brain, it first became necessary to identify the truncation sites. Truncated α-synuclein species were purified from the LB-enriched, particulate fraction of DLB brain, and exact cleavage sites were determined by sequencing of tryptic peptides by LC-MS/MS (Table 2). α-Synuclein species terminating at Asp 115 (synuclein-D115), Asp 119 (synuclein-D119), Asn 122 (synuclein-N122), Tyr 133 (synuclein-Y133) and Asp 135 (synuclein-D135) were identified. All peptides terminating at Tyr 133 were phosphorylated at Ser 129, strongly suggesting that it is a major constituent of the 15 kDa phosphorylated synuclein species described earlier. The assignment of the other truncation sites to the various truncated species resolved on 2D gels was based on immunoblot analysis using antibodies with well defined recognition epitopes. Results are shown in Table 1. The presence of multiple truncations comprising a single band on a 1D gel in combination with poor resolution on 1D gels further emphasized the need for reagents that distinguish between individual truncations. As a starting point, a neo-epitope antibody to synuclein-D119 was generated.

The polyclonal antibody EL101, raised against the synuclein peptide terminating at Asp 119, specifically recognized synuclein-D119 but not full-length or another truncated species (synuclein-N122; Suppl. fig. 6). As predicted from its assignment in Table 1, the 12 kDa truncated synuclein species in LBs reacted with the neo-epitope antibody (Fig. 7) confirming its identity as synuclein-D119. EL101 was also used to immunostain sections of brain from patients with various synucleinopathies (DLB, MSA, and familial PD caused by an A53T α-synuclein mutation) and a control individual (Fig. 8). While only diffuse background staining was observed in the control sections (Fig. 8A), EL101 extensively stained LBs and LNs in DLB brain (Fig. 8B) similar to that observed with the α-synuclein antibody EL48 (Fig. 8C). There was no obvious decrease in staining intensity when EL101 was pre-incubated with full-length recombinant α-synuclein (Fig. 8D), whereas pre-absorption of EL101 with its antigenic peptide abolished staining of LBs and LNs (Fig. 8E). These results indicate that the staining observed with EL101 is specific for synuclein-D119. Staining of the familial PD case with EL101 also revealed the presence of very extensive LNs (Fig. 8F and I), the predominant pathological inclusion in individuals with an A53T synuclein mutation (16, 19). In brain sections of an MSA patient, EL101 labeled abundant GCIs (Fig. 8G and H). The widespread presence of synuclein-D119 in LBs, LNs and GCIs suggests that not only does the synuclein composition of LB preparations reflect that of LBs in situ but moreover that synuclein-D119 is present in synuclein positive inclusions in multiple synucleinopathies and does not appear to be restricted to a subset of these pathological inclusions.

The levels of synuclein-D119 in LBs and in brain cytosol were compared on 1D immunoblots probed with EL101 (Fig. 3C). Synuclein-D119 was detected as a 12 kDa protein in all samples. A small amount of higher molecular weight EL101 immunoreactive species were also present, but only in LBs. The molecular weight of these species (25, 37 and 100 kDa) correspond to multimers of 2, 4 and 8 molecules of synuclein-D119 or mixed multimers of synuclein-D119 and full-length synuclein. A minor portion of the synuclein-D119 may have also undergone a post-translational modification (i.e. ubiquitination) causing an increase in molecular weight as described below. Even when taking all these synuclein-D119 species into account, synuclein-D119 did not appear to be highly enriched in LBs.

Because there appeared to be some variability in the amount of the truncations in LB preparations between patients (Suppl. Fig. 2), additional patients were examined to further address the question of whether synuclein-D119 was enriched in LBs. In Suppl. fig. 4C, EL101 immunoreactivity was examined in the soluble and LB enriched particulate fraction of 4 control and 5 DLBD patients. The variability in the amount of EL101 immunoreactivity in the samples did not correlate with either postmortem interval or patient age (Suppl. Table 1). When the immunoreactivity was quantitated and compared in Fig. 4C, a small but non-significant increase of synuclein-D119 was observed in the DLB versus the control particulate fraction. The extent of accumulation
was similar to that of full-length synuclein (Fig. 4A) indicating that although synuclein-D119 was present in LBs, it was not highly enriched, in contrast to that observed with P-S129 synuclein (Fig. 4B).

**High molecular weight species** - LBs contain a set of unique, high molecular weight α-synuclein species (Fig. 1; Suppl. Fig. 2). Based on 2D immunoblots of LB preparations, the amount of high molecular weight species varied between preparations (Suppl fig. 2) but nonetheless comprised only a small portion of the total synuclein in LBs. The 3 major species (apparent molecular mass of 24, 32 and 42 kDa) were resolved on both 1D and 2D gels and were immunoreactive for ubiquitin (Fig. 9). Sampathu et al (4) reported ubiquitinated synuclein species of approximately the same size. Based on the apparent molecular mass of synuclein (Mr 16 kDa) and ubiquitin (Mr 8 kDa; 20) the high molecular weight species may be accounted for as the mono-, di-, and tri-ubiquitinated forms of full-length synuclein. The basic shift in pI is also consistent with the addition of ubiquitin to synuclein. Non-ubiquitinated synuclein oligomers of 25-35 kDa, present in LBs but only in very small, variable amounts, do not display the dramatic pI shift signifying ubiquitination (Fig. 1; Suppl. fig. 2). In all LB preparations, the overall amount of ubiquitinated, as well as oligomeric, synuclein is less than that of the full-length synuclein in LBs. Despite the low level of ubiquitinated synuclein, α-synuclein is the major ubiquitinated protein of LBs. The only other protein in LBs detected with the ubiquitin antibody was unconjugated ubiquitin monomer and multimers at pI 6.6, the theoretical pI of ubiquitin (Fig. 9B).

The major ubiquitinated synuclein species in LBs are phosphorylated at Ser 129 based on their reactivity with the P-S129 specific antibody 11A5 (Fig. 2) as well as their co-purification with P-S129 synuclein from the DLB brain (Fig. 10). During the purification of synuclein from the particulate fraction of DLB brain, the P-S129 synuclein was separated from the bulk of non-phosphorylated synuclein by anion-exchange chromatography (Fig. 10A and B) and was further purified by capture and elution from 11A5 conjugated resin (Fig. 10C). All of the high molecular weight synuclein species co-fractionated with P-S129 synuclein on the anion-exchange column and co-eluted with P-S129 synuclein from the immunoaffinity column with virtually none in the flow-through containing non-phosphorylated synuclein (Fig. 10C). Thus, ubiquitinated synuclein is predominantly phosphorylated at Ser 129.

To identify sites of ubiquitination, the three high molecular weight species in the purified P-S129 synuclein preparation were resolved by SDS PAGE (Fig. 11). Each of the high molecular weight bands was excised from the Coomassie stained gel, digested with trypsin and analyzed by LC-MS/MS. Ubiquitinated Lys residues in synuclein peptides were identified by the addition of 114 Da, the mass of a diglycine peptide produced from tryptic digestion of ubiquitin. Two diglycine modified Lys residues, Lys 12 and 21, were detected on the 24 kDa synuclein species (band 1). The apparent molecular mass of band 1 corresponds to that of synuclein (Mr 16 kDa) with the addition of a single ubiquitin (Mr 8 kDa). The tryptic peptides recovered from band 1 are consistent with a mixture of synuclein monomers singly ubiquitinated at one of two sites. An additional diglycine modified residue, Lys 23 was detected from analysis of the 32 kDa (band 2) species. A single diglycine modified Lys was also detected in the peptide encompassing amino acids 11-23 of synuclein but it was not possible to distinguish whether Lys 12 or 21 of this peptide was modified. With an overall molecular mass corresponding to the addition of 2 ubiquitins, the 42 kDa species is either mono-ubiquitinated on two of these sites or is composed of a mixture of synuclein molecules including one with a di-ubiquitin chain on a single site. Analysis of the 42 kDa synuclein species (band 3) showed evidence of polyubiquitin chains. Although no α-synuclein peptides for this 42 kDa synuclein species were detected by LC-MS/MS (despite positive immunoreactivity on western blots), ubiquitin peptides were identified including one with a diglycine modified Lys 48. Lys 48 is recognized as a residue of ubiquitin which can form an isopeptide bond with Gly 76 of another ubiquitin molecule to create a polyubiquitin chain (21, 22).

In addition to being found in DLB brain, ubiquitinated synuclein species were also found in other synucleinopathies. High molecular weight α-synuclein species, with the size increase and pI shift characteristic of ubiquitination, were
observed in a preparation of GCI from an MSA brain and in the highly insoluble brain fraction of a Contursi kindred family member (Suppl. fig. 2F; Fig. 6). In DLB brain, the ubiquitinated synuclein was restricted to LBs. The high molecular weight species were detected only in LB preparations (Fig. 1; Suppl. fig. 2) and the LB-rich particulate fraction of DLB brain (Suppl. fig. 4B); none was detected in the soluble fraction (Suppl. fig. 4; Fig. 10C). Its presence in the particulate fraction of DLB brain is most likely due to its presence in LBs based on its insolubility (Fig. 5). Ubiquitinated synuclein is distinctly absent from normal brain, being detected neither in the soluble or particulate fractions (Figs. 1A and 5; Suppl. fig. 4). Ubiquitinated synuclein was therefore strictly associated with the pathological structure that defines synucleinopathies, the LB.

**DISCUSSION**

A comprehensive, unbiased inventory of α-synuclein in LBs isolated from DLB brain has identified a set of consistent and specific modifications to α-synuclein (the only member of the synuclein protein family observed in LBs). The modifications include phosphorylation of Ser 129, ubiquitination, and truncation at several sites between Asp 115 and the C-terminus. We have assessed abundance and enrichment of these modifications in LBs relative to that in the soluble fraction of both control and DLB patients. Furthermore, we extended our analysis to another synucleinopathy, MSA, and familial PD patients, in which a mutation in α-synuclein drives disease and accompanying pathology, in order to determine the broad physiological relevance of these modifications.

To survey the different species of α-synuclein in LBs, LBs isolated from 4 different individuals with DLB were resolved on 2D gels. Using both pan and P-S129 α-synuclein specific antibodies, we established that P-S129 α-synuclein is the most abundant modified form of synuclein in LBs. These results agree well with the previous identification of P-S129 α-synuclein in detergent-insoluble fractions from DLB brain and in LBs and LNs (10). In addition our data also demonstrates that a small amount of P-S129 α-synuclein is present in the soluble fraction of both control and DLB brain. Quantitation of total and P-S129 α-synuclein levels with novel pan and P-S129 α-synuclein ELISAs and by western blot analysis confirms the high degree of enrichment of the phosphorylated species in LBs over that in the cytosol. No other “priming” modification(s) appears to be required for the phosphorylation since we have obtained complete coverage of the phosphorylated α-synuclein by MS/MS mapping, without detection of any additional modifications apart from the ubiquitous N-terminal acetylation. N-terminal acetylation of cytosolic proteins is a common occurrence, especially for those proteins in which the initiating Met residue is retained (23).

The abundance and enrichment of P-S129 synuclein in LBs compared to that in the cytosol is consistent with a model in which cytosolic P-S129 α-synuclein preferentially deposits into LBs. The small decrease in cytosolic P-S129 α-synuclein in DLB versus normal brain, although not statistically significant, may reflect the shift of P-S129 α-synuclein from the cytosol to the LB. In further support of this hypothesis, P-S129 α-synuclein has been shown to be more prone to aggregate in vitro (10), and phosphorylation of α-synuclein enhances inclusion formation in SY5Y cells (24). Although it is possible that synuclein may be phosphorylated once it has deposited into the LB, the observed levels of P-S129 synuclein requires virtually complete access of Ser 129 of synuclein in the fibrillar deposits to the phosphorylating kinase, and complete protection of the phosphorylated Ser from cellular phosphatases. These stringent requirements make this scenario seem less likely than the simpler model of preferred deposition of P-S129 synuclein.

A small portion of the synuclein in LBs is ubiquitinated, in agreement with the results of Hasegawa et al (25) and Nonaka et al (26). Immunoblots of 1D and 2D PAGE gels stained with antibodies to synuclein and ubiquitin show that the major ubiquitinated species correspond in molecular mass to α-synuclein with one to three ubiquitins attached. Hasegawa et al (25) reported a ubiquitinated Lys in the peptide comprising amino acid residues 6-13 of synuclein isolated from the insoluble brain fraction of an MSA patient although the exact modified Lys residue was not identified. In this study, ubiquitination of
specific Lys residues was inferred from the presence of diglycine modified Lys residues detected in tryptic digests of synuclein by MS analysis. Diglycine modification of Lys residues 12, 21 and 23 of α-synuclein were identified indicating multiple sites of ubiquitination. In addition, this is the first report that at least some of the ubiquitin molecules are present as polymer with individual ubiquitin molecules linked via Lys 48. Polyubiquitin chains linked via Lys 48 are preferentially targeted to the proteasome while linkage through other ubiquitin residues, e.g., Lys 29 and 63, have other functions (21, 22).

The ubiquitinated synuclein in LBs is predominantly phosphorylated at Ser 129, as also seen by Tofaris et al (27) and Hasegawa et al (25). The preferential ubiquitination of P-S129 α-synuclein may reflect the fact that it is the most abundant form of synuclein in the LB (10). Alternatively phosphorylation may be the trigger for ubiquitination; however we did not detect ubiquitination on P-S129 α-synuclein in the soluble fraction of DLB brain nor in control brain. The absence of ubiquitinated synuclein outside LBs strongly suggests that the observed ubiquitination occurs after the P-S129 α-synuclein has deposited into LBs. It is possible that ubiquitination may affect the solubility of the deposited α-synuclein, but since the majority of insoluble α-synuclein in the LB is not ubiquitinated, ubiquitination alone is unlikely to drive the formation of the LBs.

α-Synuclein is the major ubiquitinated protein detected in isolated LBs from DLB brain. 2D immunoblots probed with a ubiquitin antibody detected only α-synuclein, although other ubiquitinated proteins may be present at much lower levels than the ubiquitinated α-synuclein. The failure to detect high levels of a variety of ubiquitinated proteins in LBs raises the possibility that the LB does not form as a result of inhibition or breakdown of the proteosomal degradation pathway. A variety of abundant, ubiquitinated proteins would be expected to accumulate in this case (28). The question of the function of the ubiquitination therefore remains unanswered. It is possible that the neuron attempts to clear the LB by ubiquitinating synuclein and targeting it to the proteosome, which would be consistent with the Lys 48 isopeptide bond found between ubiquitin molecules in synuclein preparations. Synuclein with a chain of four or more ubiquitins, the critical number for proteosomal recognition (22), may have already been cleared from the LB leaving behind synuclein with limited ubiquitination.

LBs contain a distinct profile of C-terminally truncated α-synuclein species (10, 11.5, 12, 12.5, 13 and 15 kDa) all with intact N-termini. The precise sites where synuclein was cleaved to generate the 11.5, 12, 12.5, and 15 kDa truncated species were identified by LC-MS/MS analysis of α-synuclein purified from the insoluble fraction of DLB brains. The truncation sites of the 12 and 12.5 kDa species, Asp 119 and Asn 122 respectively, were also recently identified by Li et al (29). A neo-epitope antibody to synuclein-D119 reacted with species of the appropriate size on immunoblots of isolated LBs confirming that synuclein-D119 is present in LBs. Furthermore, on brain sections from individuals with DLB and familial PD (A53T synuclein mutation), the neo-epitope antibody to synuclein-D119 immunostained the full range of Lewy pathologies from LBs to LNs.

Like P-S129 α-synuclein, the 12, 12.5 and 15 kDa truncated species were consistently found outside the LB in DLB brain and in normal brain, largely as soluble proteins, suggesting that they are a product of normal synuclein metabolism. The 10 kD truncation is detected in the cytosol of at least some individuals. The 11.5 and 13.5 kDa synuclein species differ from the other truncated species in that they are restricted to LBs suggesting that they may be generated there. Proteolytic digestion of fibrillar synuclein versus soluble synuclein has been reported to generate novel truncated synuclein species (30, 31). In this scenario proteases cleave exposed regions of synuclein leaving the filamentous core. Alternatively, the location of the LB in the neuron may result in the exposure of associated synuclein to proteases with which it normally has no long-term contact.

The role of the C-terminally truncated species in the disease remains to be established. C-terminally truncated synuclein has been reported to aggregate more readily than full-length α-synuclein suggesting that the truncated species could drive LB formation (32-34). The modest levels of truncated synuclein and their lack of
enrichment in the LBs, however, indicate that LB formation is not the result of a simple amyloidosis of truncated synuclein. The truncations may instead drive LB formation by acting as a seed for P-S129 α-synuclein aggregation. Although this has not been experimentally tested, other studies have demonstrated that synuclein truncated at various sites between amino acids 102 and 123 enhance aggregation of full-length synuclein both in vitro and in cells (29, 32, 35). Consistent with the seed hypothesis, synuclein-D119 is widely present in LBs, LNs and GCIs in different synucleinopathies (DLB, familial PD and MSA) as would be expected for the inclusion nidus.

It is also possible that the truncated species may be generated in the LBs, the trigger for initial proteolysis being phosphorylation at Ser 129, the most abundant synuclein modification in LBs. Alternatively, phosphorylation at Ser 129 may drive truncation at potentially pathogenic positions. This possibility is consistent with the observation that all of the synuclein-Y133 detected by LC/MS/MS is phosphorylated at Ser 129. Phosphorylation-driven proteolysis may also occur in the cytosol, which would explain why the soluble fraction of DLB brain had both lower levels of P-S129 synuclein and synuclein-D119; the less P-S129 synuclein available for proteolysis, the less truncated synuclein produced.

In summary, the α-synuclein composition in LBs differs from that in the cytosol of neurons. Rather than a gross accumulation of cytosolic synuclein into LBs, a particular modified synuclein species, P-S129 synuclein, preferentially and consistently accumulates in LBs and therefore may drive LB formation. A specific set of C-terminal truncations is also characteristically found in the LBs although their role in LB formation is not presently known. These same two modifications, phosphorylation and truncation of α-synuclein, are also found in individuals with familial PD (A53T synuclein mutation) and MSA, thereby indicating their widespread, potentially causative role in the expanding spectrum of synucleinopathies.
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FOOTNOTES

1The abbreviations used are: LB, Lewy body; LN, Lewy neurite; DLB, dementia with Lewy bodies; MSA, multiple system atrophy; PD, Parkinson’s disease; MS, mass spectrometry; LC, liquid chromatography; HPLC, high pressure liquid chromatography; IEF, isoelectric focusing; 2D, two-dimensional; 1D, one-dimensional; BSA, bovine serum albumin; HRP, horseradish peroxidase; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DAB, 3, 3’ diaminobenzidine; XCorr, cross correlation score; P-S129, phosphorylated Ser 129; synuclein-D115, synuclein truncated at Asp 115; synuclein-D119, synuclein truncated at Asp 119; synuclein-N122, synuclein truncated at Asn 122; synuclein Y-133, synuclein truncated at Tyr 133; synuclein-D135, synuclein truncated at Asp 135.
2Wei Ping Gai, personal communication
FIGURE LEGENDS

Fig. 1. α-Synuclein in LBs and the soluble fraction of brain.  (A-D) Immunoblots of soluble brain protein (A, C) of a control patient and a LB preparation (B, D), resolved by 2D PAGE using either pH 4-7 (A, C) or pH 3-10 (B, D) IEF gradients, were probed with the α-synuclein antibody Syn-1. White asterisks in (A) and (C) mark full-length synuclein, unmodified except for N-terminal acetylation. Boxes in (B) highlight differences between soluble and LB α-synuclein: acidic modifications of full-length monomer (solid), C-terminally truncated species (dashed), and high molecular weight species (dotted). (C) and (D) show enlargements of the corresponding regions in (A) and (B), respectively, with apparent molecular weights of synuclein spots indicated. The soluble protein was prepared from the brain of a control patient (91/290) and the LBs were isolated from a DLB patient (P50). pI values and positions of molecular weight standards are indicated. (E) A LB preparation from DLB brain (DLB), resolved on 2D gels in panels B and D, and a mock LB preparation from control brain (Control) were probed on immunoblots for α-synuclein using the antibody Syn-1. An equivalent amount of protein (3 ug) was loaded in each lane. The apparent molecular weights of full-length synuclein and its C-terminal truncations are shown. The 12 and 12.5 kDa truncated species were not resolved on the 1D gel but were separated on the 2D gels as shown in A-D. Positions of molecular weight standards are indicated on the right margin. The mock LB preparation was performed on brain tissue from control patients (91/290 and 91/204).

Fig. 2. α-Synuclein in LBs and GCIIs is highly phosphorylated at Ser 129.  (A-D) Immunoblots of LB (A, C) and soluble (B, D) brain proteins were developed with Syn-1 (A and B) or the P-S129 synuclein specific antibody 11A5 (C, D). Insets in (A) and (C) show enlargements of the 16 kD and 15 kD α-synuclein species. Note that panels A and C, and B and D, show serial probes of the same respective blots. The order of exposure to the antibodies did not affect the results. Arrows mark identical spots, and asterisks label Syn-1 immunoreactive spots that were absent on 11A5 reprobes, i.e. non-phosphorylated α-synuclein. The brain preparations, from patients 91/290 and P50, were resolved on pH 3-10 IEF gradients. pI values and positions of molecular weight standards are indicated. (E) ELISA quantitation of total and P-S129 α-synuclein from multiple LB preparations from 4 patients (P36, P48, P50 and P52), a GCI preparation from an MSA patient (P28), and the soluble brain fraction from 4 control (N91/204, N91/290, N27, and N28) and 4 DLB (P23, P26, P36, and P40) patients. Although the soluble brain samples contained a measurable level of total α-synuclein, the amount of P-S129 synuclein fell below the limit of quantitation of the phospho-specific assay. Results are presented as the ratio of the signals from the two ELISA assays.

Fig. 3. Comparison of α-synuclein in the soluble fraction of control and DLB brain with that in LBs. Immunoblots of the soluble protein from control brain (lane 1, patient N290; lane 2, patient N204), DLB brain (lane 3, patient P25; lane 4, patient P40) and LBs (lane 5; patient P50) were probed with Syn-1 (A), 11A5 (B), or EL101 (C). An arrow and arrowhead mark full-length synuclein and synuclein-D119 respectively. Samples loaded in each lane contain a constant amount (12 ng) of α-synuclein as determined by ELISA. Note that the high molecular weight (>100 kDa) immunoreactive bands in panel B are due to non-specific cross-reactivity with the 11A5 antibody. Bands of the same molecular weight are detected with 11A5 in cells transfected with α-synuclein but the intensity of the bands does not change with the expression level of α-synuclein. pI values and positions of molecular weight standards are indicated.

Fig. 4. Quantitation of the α-synuclein species in control and DLB brain. Full-length α-synuclein, P-S129 synuclein and synuclein-D119 in the soluble and particulate fractions of 4 control and 5 DLB brains were examined on immunoblots (Suppl. fig. 4). The blots were scanned and the amount of each of the 3 synuclein species was quantitated relative to a standard on each blot by densitometry. Values plotted are
the optical density (OD) of the α-synuclein species, divided by that of the respective standard. (Ctrl)-
control, (sol)- soluble, (partic)- particulate. Error bars represent the SEM. (*) P<0.05

Fig. 5. **P-S129 α-synuclein is enriched in the highly insoluble fraction of DLB brain.** The particulate
fractions of DLB and control brain (patients P36 and 91/182) were either solubilized in Laemmli sample
buffer (total) or serially extracted with equal volumes of 0.1% Triton (Triton), 8 M urea (urea), and IEF
lysis buffer (UTC). A constant volume of the 100,000g supernates of each extract from DLB (lanes 1-4)
and control (lanes 5-8) samples were analyzed on immunoblots probed with Syn-1 (A) or 11A5 (B).

Fig. 6. **Serial extractions of the detergent insoluble fraction of control and Contursi brain.** The
Triton and NP40 insoluble fraction of control and Contursi brain was serially extracted with Tris-buffered
sucrose (TBS), urea, and urea/SDS. Extractions were followed by centrifugation at 100,000g for 30
minutes. Immunoblots of the resulting supernates (10 µg/lane) from the control (lanes 1-5) and Contursi
brain (lanes 6-10) are shown in lanes 1 and 4 (TBS), lanes 2 and 5 (urea extract) and lanes 3 and 4
(urea/SDS extract). Immunoblots were probed with Syn-1 to detect α-synuclein (A) and 11A5 to detect
P-S129 synuclein (B). An arrow indicates the position at which recombinant α-synuclein migrates. Both
panels A and B are photographic composites, each prepared from the same exposure of a single
immunoblot probed with the specified antibody.

Fig. 7. **Synuclein-D119 is present in LBs.** An immunoblot of LB proteins resolved by 2D PAGE were
serially probed with Syn-1 (A) to detect total α-synuclein species and EL101 (B) to detect synuclein-
D119. Only those regions of the blots containing the C-terminally truncated species are shown. Asterisks
identify corresponding immunoreactive spots on the vertically aligned blots. The apparent molecular
weights of the truncated α-synuclein species were extrapolated from molecular weight standards. EL101
detects only the 12 kDa truncated synuclein species. LBs were prepared from DLB patient AD7.

Fig. 8. **Synuclein-D119 is present in LBs, LNs and GCIs.** Brain sections from the midfrontal cortex
of a control individual (A), DLB patients (B, D, E), an individual with an A53T α-synuclein mutation (F, I)
and an MSA patient (G, H) were stained with the synuclein-D119 specific antibody EL101. DLB
brain was also stained with the α-synuclein antibody EL48 (C). Arrows point to LBs, or GCIs in MSA
brain (G), and arrowheads denote LNs. To test specificity, EL101 was pre-absorbed with either full-
length recombinant α-synuclein (D) or the synuclein-D119 peptide antigen (E) prior to staining DLB
brain. In sections from the individual with an A53T synuclein mutation, EL101 labeled both small LNs
in upper cortical layers I-III (F) and larger LNs in the deeper Layer VI (I). In MSA brain, scattered GCIs
and LNs were labeled in grey matter (G) while numerous GCIs were stained in white matter (H). The
scale bar in all panels is equivalent to 100 µm. Although sections from multiple control, DLB and MSA
brains were stained for α-synuclein and synuclein-D119, only representative sections from the following
patients are shown: control individual N27 (A), DLB patients P36 (B, C) and AD22 (D, E), and MSA
patient P28 (G, H).

Fig. 9. **High molecular weight α-synuclein species are ubiquitinated.** An immunoblot of LB proteins,
resolved by 2D PAGE, was serially probed with the α-synuclein specific antibody Syn-1 (A) and an anti-
ubiquitin antibody (B). Lines connect corresponding spots reacting with both antibodies. A minor set of
lower molecular weight, ubiquitinated synuclein species trails from each of the major species toward the
basic end of the 2D gel. This same pattern is reminiscent of the C-terminally truncated synuclein seen in
LBs. Lack of immunoreactivity with the C-terminal antibody 8A5 and EL48 (data not shown) confirms
that the minor species are C-terminally truncated. An arrow marks a spot at pI 6.6 that is consistent with
the pI and molecular mass (Mz 8 kDa) of ubiquitin. Higher molecular weight spots of the same pI are
likely to be ubiquitin multimers. LBs were isolated from P52. pI values and positions of molecular
weight standards are indicated.
Fig. 10. Purification of P-S129 synuclein from the particulate fraction of DLB brain by anion-exchange and immunoaffinity chromatography. (A and B) Protein from the particulate fraction of DLB brain was solubilized in UTC buffer, and first separated on a Q-Sepharose anion-exchange column. Shown are the extract applied to the column (load), the column flow-through (FT), low salt wash (wash) and serial fractions collected during elution (4-43). Immunoblots of the samples were probed for total α-synuclein (A) and P-S129 synuclein (B) using the antibodies Syn-1 and 11A5 respectively. The elution fractions were pooled based on their content of truncated (pools 1-3) or phosphorylated synuclein (pool 4) for further purification. (C) Pool 4 from the Q-Sepharose column was applied to an 11A5-conjugated Sepharose column, and the column flow-through (lane 3, P-), largely depleted of P-S129 synuclein, and eluate (lane 2, P+) were collected. Synuclein from the soluble fraction of the brain is shown in lane 1. Immunoblots of the samples were probed with Syn-1 and 11A5 as indicated.

Fig. 11. Identification of ubiquitination sites on P-S129 synuclein and a polyubiquitin linkage. P-S129 synuclein purified from the particulate fraction of DLB brain was resolved by SDS PAGE and stained with colloidal Coomassie Blue. The high molecular weight synuclein species labeled as bands 1 (27 kDa), 2 (34 kDa) and 3 (44 kDa) were excised, digested with trypsin, and analyzed by LC-MS/MS. Spectra were searched for lysine residues modified by diglycine addition through their ε-amino group, an indicator of ubiquitination. The peptides of synuclein or ubiquitin with these modified lysine residues (K*) are shown. Modified Lys residues 12 and 21 from synuclein peptides were detected in band 1 and a modified Lys 23 was found in band 2. Another synuclein peptide from band 2 comprising amino acid residues 11-23 contained a modified Lys residue but it was not possible to establish whether it was Lys 12 or 21. A modified Lys 48 residue in the ubiquitin peptide containing residues 43-54 was detected in band 3. The latter results from the linkage of two ubiquitin molecules.
Table 1. C-terminally truncated α-synuclein species. Full-length and C-terminally truncated α-synuclein species identified in LBs are listed according to their apparent molecular mass on 1D gels. The C-terminal amino acid of each species was estimated based on immunoreactivity with a series of synuclein antibodies with mapped epitopes (Estimated cleavage site). The amino acids of human α-synuclein comprising the epitope are listed below each antibody. The C-terminal amino acid of the synuclein species (C-terminus) was identified by LC-MS/MS.

Not resolved from synuclein-D135 on 2D gels.

Not yet identified by MS/MS mapping; cleavage site estimated from mobility on SDS PAGE and antibody reactivity.

| M_r (kD) | C terminus | Estimated cleavage site | Immunoreactivity with synuclein antibodies |
|----------|------------|-------------------------|-------------------------------------------|
| 16       | full length | 140 (intact)            | +                                         |
| 15       | Asp 135    | 129-140                 | +                                         |
|          | Tyr 133 a  |                         |                                           |
| 13.5     | Glu 126- Ser 129 b | 126-129                  | +                                         |
| 12.5     | Asn 122    | 123-126                 | +                                         |
| 12       | Asp 119    | 120-123                 | +                                         |
| 11.5     | Asp 115    | 96-120                  | +                                         |
| 10       | Lys 96- Glu 105 b | 96-105                  | +                                         |

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aNot resolved from synuclein-D135 on 2D gels.
bPosition not yet identified by MS/MS mapping; cleavage site estimated from mobility on SDS PAGE and antibody reactivity.
Table 2. Identification of C-terminally truncated α-synuclein species. Truncated α-synuclein species, enriched by anion exchange chromatography (pool 2/3, Fig. 10A) and purified by immunoaffinity chromatography (5C12-conjugated Sepharose), were digested with trypsin and analyzed by LC-MS/MS. MS/MS spectra were searched against a database of serial C-terminal α-synuclein truncations spanning amino acids 98-140. The sequence and corresponding amino acid numbers in the human α-synuclein sequence are listed for each peptide identified. The truncated synuclein species are named according to their terminal amino acid, e.g. synuclein-D119 refers to synuclein terminating at Asp 119. # indicates phosphorylation of Ser 129. * indicates oxidation of the Met residue. MH+ and z respectively refer to the theoretical average mass of the mono-protonated peptide and the charge state of the peptide. P-values and X corr scores are a measure of the accuracy of the peptide match.

| α-Synuclein species | Amino acids | Peptide | MH+  | z   | P value  | XCorr |
|---------------------|-------------|---------|------|-----|----------|-------|
| Synuclein-D115      | 81-115      | TVEGAGSIAATGFVKKDQLGKNEEGAPQEGILED | 3531.8 | 3   | 3.34E-03 | 3.66  |
| Synuclein-D119      | 97-119      | KDQLGKNEEGAPQEGILEDMPVD          | 2513.7 | 2   | 9.62E-09 | 3.45  |
| Synuclein-N122      | 98-122      | DQLGKNEEGAPQEGILEDMPVDPDN        | 2711.8 | 2   | 3.25E-08 | 3.90  |
|                     | 97-133      | KDQLGKNEEGAPQEGILEDMPVDPDNEAYEMS#EGY | 4206.3 | 3   | 9.90E-03 | 3.02  |
| P-S129 Synuclein-Y133| 98-133     | DQLGKNEEGAPQEGILEDMPVDPDNEAYEM*PS#EGY | 4094.1 | 3   | 6.80E-05 | 2.55  |
|                     | 98-133      | DQLGKNEEGAPQEGILEDMPVDPDNEAYEMS#EGY | 4094.1 | 3   | 1.05E-06 | 2.70  |
| Synuclein-D145      | 98-135      | KDQLGKNEEGAPQEGILEDMPVDPDNEAYEMPSEEGYQD | 4369.5 | 3   | 4.89E-03 | 4.77  |
Figure 1

Soluble

LBs

E)

DLB Control

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**Figure 2**

Relative levels of P-S129 and total α-synuclein

![Image of protein blots and graph showing relative levels of P-S129 and total α-synuclein](image)

- **E)** Relative levels of P-S129 and total α-synuclein

The graph shows the phospho/total ELISA ratio for various samples, including p36, p38, p50, p52, MSA, soluble DLB, and soluble Control.
Figure 3

A) Syn-1

B) 11A5

C) EL101

Soluble

Ctrl Ctrl DLB DLB

Soluble

Ctrl Ctrl DLB DLB

Soluble

Ctrl Ctrl DLB DLB

LB

LB

LB

1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

200

150

100

50

37

25

15

10
Figure 4

A) 
**Full-length α-Synuclein**

B) 
**P-Ser129 α-Synuclein**

C) 
**α-Synuclein-D119**
Figure 5

A) Syn-1

|    | total | Triton | urea | UTC |
|----|-------|--------|------|-----|
| DLB |       |        |      |     |
| control |       |        |      |     |

B) 11A5

|    | total | Triton | urea | UTC |
|----|-------|--------|------|-----|
| DLB |       |        |      |     |
| control |       |        |      |     |

Legend:
- Triton: Triton X-100
- urea: Urea
- UTC: Untreated
### Figure 6

#### A) Total (Syn-1)

|       | Control | Contursi |
|-------|---------|----------|
| TBS   |         |          |
| Urea  |         |          |
| UTC   |         |          |

#### B) P-S129 (11A5)

|       | Control | Contursi |
|-------|---------|----------|
| TBS   |         |          |
| Urea  |         |          |
| UTC   |         |          |
Figure 7

A) [Image]

B) [Image]
Figure 10

A) Total α-Synuclein

B) P-S129 α-Synuclein

C)
**Figure 11**

### Ubiquitin (K48)

| Amino Acids | Ubiquitin Peptide        | MH+  | z   | P Value | XCorr |
|-------------|--------------------------|------|-----|---------|-------|
| 43-54       | LIFAGK*QLEDGR            | 1460.7 | 2  | 3.76E-07 | 3.78  |

### α-Synuclein (K12, K21, K23)

#### α-Synuclein (K12, K21)

| Amino Acids | α-Synuclein Peptides       | MH+  | z   | P Value   | XCorr |
|-------------|---------------------------|------|-----|-----------|-------|
| 11-21       | AK*EGVVAAAEK*TK           | 1415.7 | 2  | 2.66E-02  | 3.34  |
| 11-23       | AKEGVVAAAEK*TK            | 1415.7 | 2  | 2.66E-02  | 3.34  |

#### α-Synuclein (K12, K21, K23)

| Amino Acids | α-Synuclein Peptides       | MH+  | z   | P Value  | XCorr |
|-------------|---------------------------|------|-----|----------|-------|
| 11-23       | AKEGVVAAAEK*TK            | 1415.7 | 2  | 1.88E-07 | 3.89  |

#### Ubiquitin (K48)

| Amino Acids | Ubiquitin Peptide        | MH+  | z   | P Value  | XCorr |
|-------------|--------------------------|------|-----|----------|-------|
| 43-54       | LIFAGK*QLEDGR            | 1460.7 | 2  | 3.76E-07 | 3.78  |

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*Note: The table entries represent the peptide sequences, mass-to-charge ratio (MH+), number of observed peptides (z), significance level (P Value), and confidence (XCorr).*
Phosphorylation of Ser 129 is the dominant pathological modification of α-synuclein in familial and sporadic Lewy body disease
John P. Anderson, Donald E. Walker, Jason M. Goldstein, Rian de Laat, Kelly Banducci, Russell J. Caccavello, Robin Barbour, Jiping Huang, Kristin Kling, Michael Lee, Linnea Diep, Pamela S. Keim, Xiaofeng Shen, Tim Chataway, Michael G. Schlossmacher, Peter Seubert, Dale Schenk, Sukanto Sinha, Wei Ping Gai and Tamie J. Chilcote

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