Urinary 8-hydroxydeoxyguanosine levels as a biomarker for progression of Parkinson disease

Abstract—8-Hydroxydeoxyguanosine (8-OHdG) has been used to evaluate oxidative stress. The authors investigated urinary 8-OHdG levels in 72 patients with Parkinson disease (PD) and in normal and disease control groups. The mean urinary 8-OHdG increased with the stage of PD and was not influenced by the current dose of DOPA. Our results suggest that urinary 8-OHdG is a potentially useful biomarker for evaluating the progression of PD.

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Oxidative damage to DNA is thought to be involved in aging and various diseases. Reactive oxygen species (ROS) including hydroxyl radical and H_2O_2 react with guanine residues in DNA and produce 8-hydroxydeoxyguanosine (8-OHdG). Conversely, oxidized DNAs are continuously repaired to prevent mutagenesis, and the excised 8-OHdG is excreted in the urine, which is considered a key biomarker of oxidative DNA damage.

Although the primary cause of Parkinson disease (PD) is still unknown, oxidative stress and mitochondrial respiratory failure are implicated in the loss of dopaminergic neurons. Indeed, several lines of evidence implicate enhanced oxidative stress in the pathogenesis of PD, dopamine being one of the main candidates. The beneficial effects of levodopa in the treatment of PD are beyond doubt; however, the controversy about its accelerating effects on the neurotoxic process is still under discussion.

Recently, urinary levels of 8-OHdG were measured in patients with cancer and diabetes mellitus to evaluate the clinical stage or the response to therapy.[1,2] In PD, there is an apparent selective increase in 8-OHdG levels in the substantia nigra.[3] Moreover, the 8-OHdG levels in serum and CSF are increased in these patients.[4] Based on this background, we postulated that if positive, urinary 8-OHdG could be a good biomarker for PD.

Methods. We studied 72 patients with PD (mean age 67.3 ± 1.6 years, ± SEM, range 47 to 88), 16 patients with multiple system atrophy (MSA) (age 63.7 ± 1.7 years, range 51 to 79), and 48 normal controls (age 57.5 ± 0.6 years, range 41 to 85). Diagnosis of MSA was based on the criteria of Gilman et al.[5] and that of PD on those of Calne et al.[6] PD was classified into five stages[7] (table). Smokers and obese subjects were excluded from the study because these factors influence urinary 8-OHdG. Except for one patient, patients with MSA could not walk alone and were classified as stage V of Yahr classification. The mean disease duration in MSA patients was 7.8 ± 1.4 years. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine.

Urinary 8-OHdG concentrations were measured using an ELISA using a monoclonal antibody specific for 8-OHdG (Nippon Yushi, Tokyo, Japan). Urine samples were obtained from each individual in the morning (between 9 to 12 AM) and immediately stored at −80 °C. Patients and controls avoided physical activity in the last 24 hours before urine sampling. In hospitalized patients with an indwelling urinary catheter, we clamped the catheter in the morning and collected urine samples after a fixed period of time. Urine samples were centrifuged at 1000*g* for 15 minutes at 4 °C and the supernatant was used for 8-OHdG measurement. ELISA was carried out in triplicate and in a blinded fashion, and the average value was used for statistical analysis. The sensitivity of ELISA ranged from 0.5 to 200 ($\times 10^{-6}$ mg/dL). We also measured urinary creatinine (mg/dL) and 8-OHdG values were expressed relative to urinary creatinine [urinary 8-OHdG/creatinine] ($\times 10^{-6}$) to adjust for muscle mass. For statistical analysis, the Student *t*-test was used for paired comparisons. All data were expressed as mean ± SEM. A p value less than 0.05 denoted a significant difference.

Results. Urinary 8-OHdG/creatinine ratio ranged from 6.61 to 23.18 in normal controls, 11.90 to 68.69 in PD, and 7.76 to 30.81 in MSA. The mean ratio of patients with PD was higher than that of age-matched control subjects ($p < 0.01$) and patients with MSA ($p < 0.05$, figure 1B. The ratio correlated with age in normal subjects ($r = 0.61$, $p < 0.01$; figure 2A but not in patients with PD ($r = 0.37$, $p = 0.62$; figure 2B.

The urinary 8-OHdG/creatinine ratio for each PD stage was Stage I: 15.0 ± 1.1 (range 11.90 to 23.02), Stage II: 22.9 ± 1.8 (range 13.47 to 32.00), Stage III: 30.2 ± 3.2 (range 20.53 to 50.11), Stage IV: 36.9 ± 2.3 (range 26.72 to 51.00), and Stage V: 46.6 ± 2.0 (range 39.76 to 68.69). The ratio increased significantly with the progression of the disease ($p < 0.01$, for Stages I and II, Stages II and IV, and Stages IV and V; figure 1A. There was no correlation between the current dose of levodopa and urinary 8-OHdG/creatinine ratio ($r = 0.05$, $p = 0.70$; figure 1C.

Discussion. Oxidative DNA damage is implicated in both aging and PD. Our results showed a significant correlation between urinary 8-OHdG excretion and the normal aging process. However, no such relationship was noted in patients with PD. These results suggest the involvement of other causes of oxidative damage in patients with PD.

Our results showed high urinary 8-OHdG concentrations in patients with PD and that these levels increased with the progression of the disease. It is noteworthy that the mean level in Stage I was not different from that of the normal controls ($p > 0.05$) and the levels in Stages I and II were not different from those of MSA. Thus, this biomarker is not suitable for early diagnosis of the disease. The cutoff

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Table *Clinical characteristics of patients with Parkinson disease and patients with MSA*

	Parkinson disease stage						
	I	II	III	IV	V	Total	MSA total
n	13	16	18	14	11	72	16
Sex, M/F	4/9	6/10	8/10	6/8	4/7	28/44	6/10
Age, y	60.5	62.8	66.2	73.9	75.5	67.3	63.7
Duration of disease, y	5.7	7.2	10.0	11.3	14.1	9.4	7.8
Levodopa, mg/d	350	422	456	571	350	435	325

MSA = multiple system atrophy.

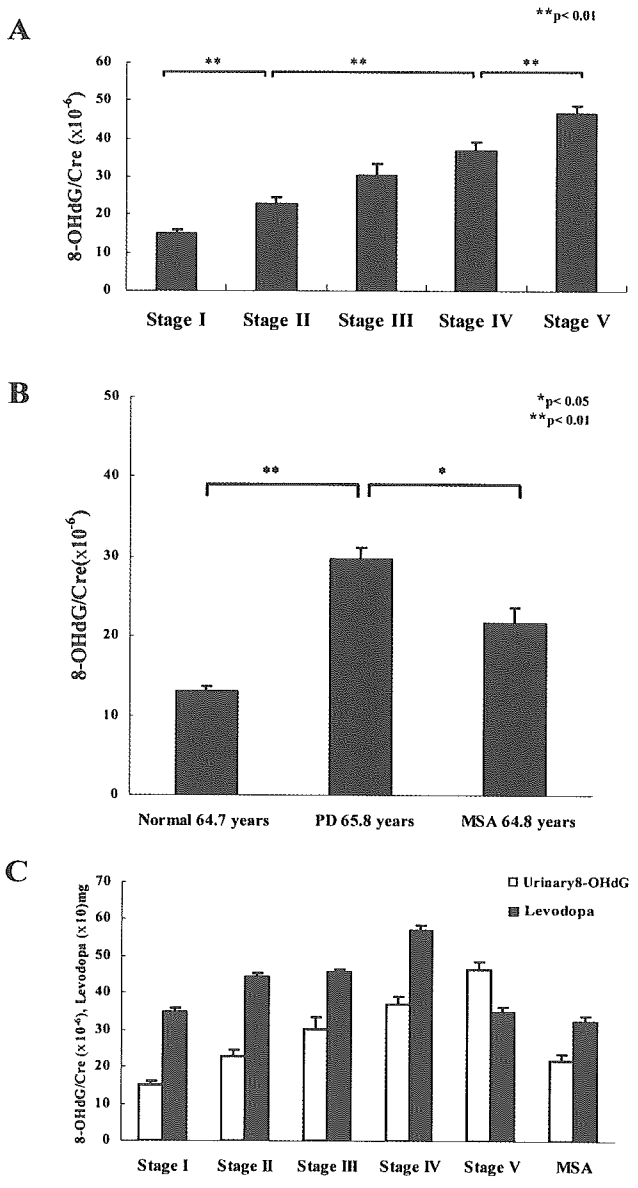

*Figure 1. (A) The mean urinary 8-hydroxydeoxyguanosine (8-OHdG)/creatinine ratio increased with the stage of Parkinson disease (PD). (B) The mean ratio was significantly higher in PD than in age-matched controls and patients with multiple system atrophy. (C) The current dose of levodopa did not influence the urinary 8-OHdG/creatinine ratio (r = 0.05, p = 0.70). Data are mean ± SEM. *p < 0.05, **p < 0.01.*

ratios were 23.18 for normal controls and 30.81 for patients with MSA. The ratio exceeded 30.81 only in patients with PD. High 8-OHdG levels in PD may be due to parkinsonism including rigidity. Rigidity may be viewed as a physical exercise load. In this regard, previous studies showed that urinary 8-OHdG levels in physically trained individuals were not different from healthy control subjects and suggested that exercise training may enhance antioxidant defense mechanisms in human skeletal muscles. It is possible that the level of systemic physical stress in patients with PD may exceed the level that could be handled by the defense mechanisms compared with continuous physical exercise.

Another potential mechanism for the increased 8-OHdG in PD is systemic mitochondrial failure[8] including brain, platelets, and skeletal muscles. Mitochondria are the most important intracellular source of ROS. In particular, systemic mitochondrial dys-

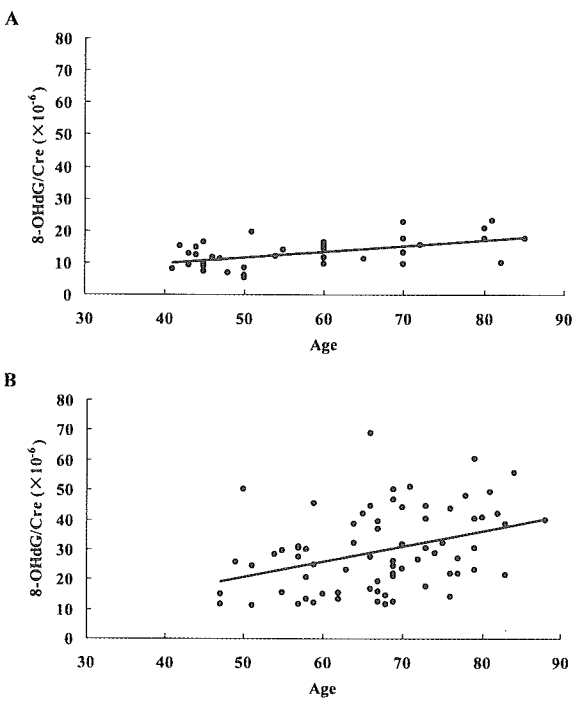

Figure 2. Urinary 8-hydroxydeoxyguanosine/creatinine (Cre) ratio of normal subjects (r = 0.61, p < 0.01) (A) and patients with Parkinson disease (r = 0.37, p = 0.62) (B).

function is considered in PD. In this regard, an apparent selective increase in 8-OHdG levels in the substantia nigra was reported. However, the characteristically high levels of the urinary 8-OHdG/creatinine ratio in our patients suggest that the mitochondrial disturbance is not solely limited to the brain but also spread over other organs such as skeletal muscles and platelets. In fact, we found no increase in 8-OHdG levels in patients without PD with cerebral thrombosis or embolism vs normal controls ($p > 0.05$ data not shown). Thus, it is unlikely that the source of high urinary 8-OHdG is the brain. The most likely organ responsible for increased 8-OHdG is skeletal muscle because the muscle tissue largely depends on an efficient oxidative energy metabolism. Previous studies reported mitochondrial dysfunction in skeletal muscles in PD.[9] We postulate that urinary 8-OHdG in PD reflects increased systemic levels of oxidative DNA damage in skeletal muscles.

Previous studies also evaluated 8-OHdG levels in various biological samples of PD patients. Mean concentrations of 8-OHdG in the serum and CSF were significantly high in PD, but the CSF concentration was generally much lower than expected considering serum 8-OHdG concentrations.[4] In this regard, it is possible that the presence of serum proteins could result in overestimation of serum 8-OHdG. Furthermore, others showed higher lipoprotein oxidation in plasma and CSF applying the lipoprotein kinetics curve.[10] However, our assay with ELISA using urinary samples allows easier evaluation of the oxidative status, and it is simple and noninvasive. We evaluate 8-OHdG levels in various stages of the disease.

Analysis of the relationship between levodopa and oxidative damage showed no correlation between 8-OHdG and the mean current dose of levodopa. Dopamine is metabolized by monoamine oxidase to generate reactive and toxic hydroxyl radicals. Our data suggest that oxidative stress is not dependent on the current dose of levodopa. Considering that 8-OHdG levels correlated with Hoehn and Yahr stage of the disease, it is possible that cumulative exposure to levodopa could influence PD progression. However, the systemic neurotoxic effects of levodopa are still not clear at present.

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Geographic and ethnic differences in frequencies of two polymorphisms (D/N394 and L/I272) of the *parkin* gene in sporadic Parkinson's disease

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Abstract

In this report, we evaluated the allele frequency of the D/N394 single nucleotide polymorphism (SNP) in exon 11 of the *parkin* gene in 200 Japanese patients with sporadic Parkinson's disease (PD) and 200 normal controls. Although the reported allele frequency of G-to-A (D/N394) is 2% in Caucasians, this SNP was not detected in Japanese patients and healthy controls. Evaluation of L/I272 polymorphism, a C-to-A transition in exon 7, showed the polymorphism in only six controls, but not in PD patients. Our results suggest that the frequencies of *parkin* polymorphisms are different among Asians and Caucasians.

Keywords: Allele frequency; TaqMan assay; Restriction fragment length polymorphism; Geographic distribution; Ethnicity; Race

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, next to Alzheimer's disease. The primary cause of PD is unknown, however, mitochondrial failure and oxidative stress are implicated in its pathogenesis [1,2]. There is general agreement that the interaction of genetic factors and environmental factors are involved in the pathogenesis of PD.

Parkin is the causative gene of early onset parkinsonism, which is characterized pathologically by neurodegeneration of dopaminergic neurons and gliosis in the substantial nigra, without Lewy body formation [3]. About 50% of cases with autosomal recessive early onset PD (AREO-PD) including autosomal recessive juvenile parkinsonism (AR-JP) cases and 18% of early onset (<40 years) sporadic PD patients were found to have mutations in the *parkin* gene [4]. Most of the *parkin* mutations are consistent with autosomal-recessive mutation [5–8], however, at times PD patients have a single *parkin* allele mutation suggestive of a dominant effect or a haplo-insufficiency [9]; such mutations might act as a risk factor for sporadic PD.

At least four polymorphisms of the *parkin* gene have been reported so far. Firstly, we reported three polymorphisms including S/N167, R/W366, and V/L380 [10]. Among them, the frequency of S/N167 in Asian population is different from that in Caucasians [10-20]—suggesting geographic and/or racial differences in the frequencies of *parkin* gene polymorphisms. Thus, it is important to determine the frequency of the *parkin* gene polymorphisms in different races. Here, we report the allele frequencies of L/I272 in exon 7 and D/N394 in exon 11 of the *parkin* gene in Japanese patients with sporadic PD since no information on the allelic frequency of both SNPs is currently available. In addition, we compare the frequencies of the *parkin* gene polymorphisms between Asians and Caucasians based on the results of the present study and those published by other investigators [10-20].

2. Subjects and methods

2.1. Subjects

The subjects of this study were 200 (82 males and 118 females) patients with sporadic PD and 200 (101 males and 99 females) control subjects free of neurodegenerative disorders. The recruited patients were from the Department of Neurology at Juntendo University School of Medicine,

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Tokyo. Some of the control subjects were the spouses of the PD patients studied. The diagnosis of PD was based on international criteria [21] and included the presence of two or more cardinal clinical features of the disease (e.g. bradykinesia, resting tremor, cogwheel rigidity, and postural reflex impairment). The onset of the disease in all PD patients studied was after the age of 40 years. The mean ± SD age at the onset of the disease was 56.7 ± 10.7 years. The mean age of control subjects was 58.3 ± 16.3 years. The study was approved by the ethics review committee of Juntendo University.

2.2. Methods

2.2.1. DNA isolation

Human genomic DNA as a template for polymerase chain reaction (PCR) was isolated from peripheral blood leukocytes according to the standard methods using a QIAamp DNA Blood Maxi Kit (50) (Qiagen, Hilden, Germany) as described previously [22]. Samples were used as soon as possible and stored at 4 °C until analysis.

2.2.2. Detection of D/N394 using TaqMan assay

We identified the polymorphism of D/N394 in the *parkin* gene using the TaqMan assay for single nucleotide polymorphism (SNP), which included two TaqMan minor groove binder (MGB) probes and two primers (forward and reverse) along with the probes. In contrast, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed in all the studies that were reported previously [10–13,15–17,19–20]. The probes and primers for allelic discrimination assays were designed by Applied Biosystem (Foster City, CA). Allelic discrimination of *parkin* D/N 394 polymorphism was performed by TaqMan assay using the ABI PRISM 7700 Sequence Detection System. The PCR reactions were carried out on 96-well plates. The SNP analyzed by the TaqMan assay was PCR amplified from genome DNA. All PCR reactions were run in triplicate, and contained about 100 ng of patient DNA, 12.5 μl of TaqMan Universal PCR Master and 0.625 μl of Allelic Discrimination Mixture. The latter consisted of 10 μM Forward primer, 10 μM Reverse primer, 5 μM TaqMan (FAM) probe, 5 μM TaqMan (VIC) probe, and Milli Q water 10.875 μl, template 1 μl, with a total volume of 25 μl. The forward primer sequences used were 5′-AACGCCTTTCCTCTTTGTTTCC-3′, the reverse primer sequences used were 5′-GAGGCTGCTTCC-CAACGA-3′, the probe sequences of FAM used were 5′-ACAGAGTCAATGAAAG-3′, the probe sequences of VIC used were 5′-AGAGTCGATGAAAGAG-3′. Appropriate negative controls were also run. TaqMan assay was performed on an ABI Prism 7700 Sequence Detection System (SDS, PE Biosystems). This reaction with allelic discrimination assays was used under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and then 35 cycles of amplification (92 °C denaturation for 15 s, and annealing/extension for 60 s at 60 °C).

After placing the PCR amplification plates in the analysis plate reader, the fluorescence data were analyzed and genotypes were classified with the post-PCR read analysis for allelic discrimination. Furthermore, we compared the frequencies of the four alleles with one another relative to the previously reported *parkin* polymorphisms [10–20].

2.2.3. Detection of L/I272 using PCR-RFLP method

Exon 7 of *parkin* gene was amplified by PCR using two primer pairs (forward primer of L/I272 were 5′-TGCTGCCTTTCCACACTGAC-3′, reverse primer of L/I272 were 5′-CATGCTAGACTTACCCACAC-3′). PCR contained template DNA 1.0 μl (100 ng), 10× PCR buffer 5.0 μl, 2 mM dNTP mixture 1.0 μl, 10 μM Sense Primer (L/I272-S) 1.0 μl, 10 μM Antisense Primer (L/I272-AS) 1.0 μl, AmpliTaq Gold 0.5 μl, and Milli Q 40.5 μl. The PCR conditions for L/I272 in exon 7 amplification were as follows: initial denaturation at 94 °C for 10 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. The polymorphism at amino acid position 272 in exon 7 was analyzed by digestion with *Tfi*I (New England BioLabs Inc.). The L/I272 polymorphism allele created a restriction site for *Tfi*I. Restriction Enzyme Digestion contained 10 μl of PCR products, 2.0 μl of 10× NEB Buffer 3, 0.5 μl of *Tfi*I (5 units/μl), and 7.5 μl of Milli Q. The reaction lasted 4 h at 65 °C. Finally, 10 μl of the reaction product was applied for 2% agarose gel electrophoresis and stained with ethidium bromide.

2.2.4. Statistical analysis

Statistical Analysis was performed using StatView-J version 4.02 (Abacus Concept, SAS, Inc., San Francisco, CA) employing the χ^2-test and Fisher's exact probability. In addition, we compared the frequencies of the *parkin* gene polymorphisms among different ethnic populations based on the results of previous studies [10–22]. Finally, we compared the frequencies of each SNPs between Asians and Caucasians using the data reported in the literature [10–20].

3. Results

Regarding SNP analysis on D/N394, the frequency of allele G was 100%, in both PD patients and the control subjects (Table 1). We also confirmed the polymorphisms of D/N394 in the *parkin* gene using the ABI PRISM 7700 Sequence Detection System and TaqMan assay (Fig. 1). These results indicated no differences in the genotypes in SNP of D/N394 between Japanese PD patients and the control subjects. Regarding the L/I272 polymorphism, all PD patients and 98.5% of the control subjects had allele C; only six normal control subjects had allele A in

Table 1
Allele frequencies of D/N394 and L/I272 polymorphisms in *parkin* in sporadic PD and normal control subjects

	Control (%)	PD (%)	Total (%)
Number of subjects	200	200	400
Number of chromosomes	400	400	800
D/N394 polymorphism			
Allele frequency			
Allele G	400 (100%)	400 (100%)	800 (100%)
Allele A	0 (0%)	0 (0%)	0 (0%)
L/I272 polymorphism			
Allele frequency			
Allele C	394 (98.5%)	400 (100%)	794 (99.2%)
Allele A	6 (1.5%)	0 (0%)	6 (0.8%)

There were significant differences in allele frequencies of L/I272 polymorphism in *parkin* between patients with Parkinson's disease (PD) and controls ($\chi^2=6.045$, df=1, $p=0.0139<0.05$).

heterozygous state (Table 1 and Fig. 3A) ($\chi^2=6.045$, df=1, $p=0.0139<0.05$). The results of RFLP on 2% agarose gel showed L/I272 polymorphism. The wild type allele was differentiated from the mutant allele after digestion with *Tfi*I (Fig. 2). We compared the frequencies of four alleles of the *parkin* polymorphisms (S/N167, R/W366, V/L380, and D/N394) with the frequencies reported in previous studies [10–20] (Fig. 3B–E, Table 2). In addition, the frequencies of the SNPs including S/N167, R/W366, V/L380, and D/N394 were significantly different between Asians and Caucasians (Table 3). Among them, the frequency of S/N167 in Asian population was higher than in Caucasians. The frequency of R/W366 polymorphism in Asian population was slightly higher than in Caucasians. In contrast, the frequencies of V/L380 and D/N394 were higher in Caucasians than in Asians.

4. Discussion

In the present study, we analyzed the frequencies of two polymorphisms of the *parkin* gene in 200 patients with sporadic PD and 200 normal control subjects. We found no statistically significant differences in the genotype and allele distribution of the D/N394 in exon 11. Our study does not provide direct evidence that *parkin* could be interrelated to sporadic PD either as a risk factor or protective factor.

Our results showed polymorphism of L/I272 in six controls, suggesting that this polymorphism could be protective against the development of PD although the frequency is too small to make a firm conclusion. Its frequency has not yet been reported in various populations. Recently, Takao et al. [23] reported the results of autopsy examination of a patient with L/I272 who showed diffuse Lewy body disease. They reported also that the L/I272 polymorphism was detected in a normal Japanese control. Therefore, this replacement cannot be considered pathogenic but rather a rare polymorphism.

A cross comparison of allele frequencies of G-to-A transition in exon 4 (S/N167) in *parkin* between Japanese and other ethnic groups showed that the Japanese

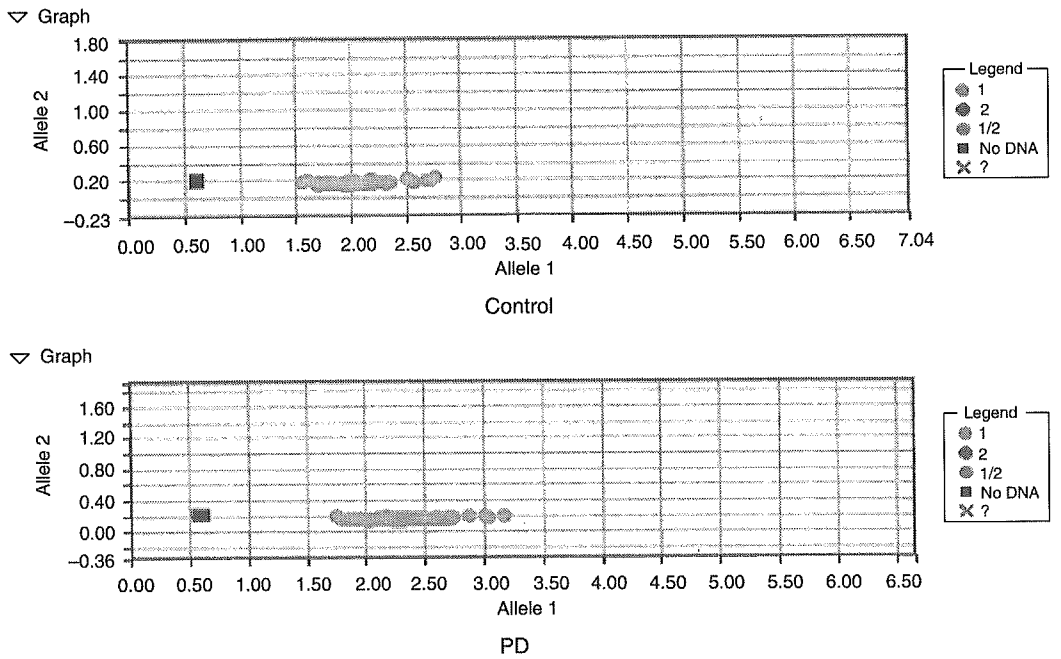

Fig. 1. Results of D/N394 polymorphism in the *parkin* gene using ABI PRISM 7700 Sequence Detection System and TaqMan assay. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter dye. Allele 1 homozygote, allele 2 homozygote, and allele 1 and 2 heterozygote are separated by reporter fluorescence. Red circles indicate allele 1 homozygote, blue circles indicate allele 2 homozygote, green circles indicate alleles 1 and 2 heterozygote, and the black square indicates no template control. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article.)

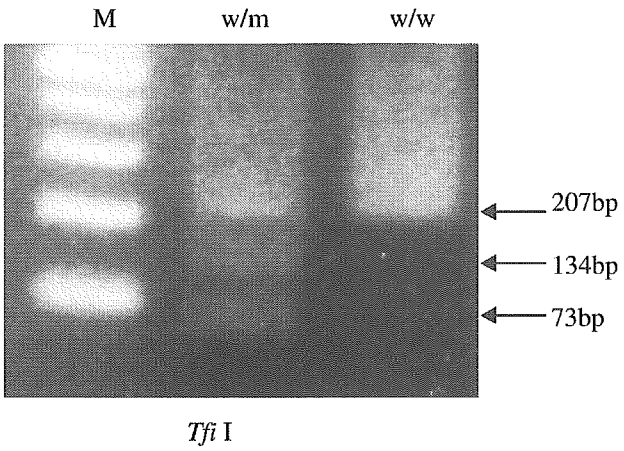

Fig. 2. Results of restriction fragment length polymorphism (RFLP) on 2% agarose gel of L/I272 polymorphism in the *parkin* gene. *Tfi*I RFLP of polymerase chain reaction (PCR) of exon 7 on 2% agarose gel. M is a 100-base pair ladder marker; w/m and w/w indicate heterozygote and wild-type homozygote, respectively.

(Wang et al. [10]; Satoh et al. [11]) were similar to the Chinese PD (China [14]; Taiwan [15]), but the frequencies in these two populations were higher than in those reported in studies from Italy [18], Spain [17], North America [13], Europe [19,20], and Finland [16]. Furthermore, the R/W366 was found in Japan [10], and China (Taiwan) [15], but not in Finland [16] or Italy [18]. The allele frequencies of V/L380 in *parkin* reported in studies from Finland [16], Italy [18], USA [13] and Europe [10–20] were higher than in studies from Japan [10] and China [15] in patients with PD. The D/N394 (G-to-A) in exon 11 has also been found in North America [13], Spain [17], Italy [18], and other European countries [10–20]. In the present study, this polymorphism (D/N394) was not found in Japanese patients with sporadic PD and healthy controls, suggesting that this polymorphism is very rare. The frequency of S/N167 polymorphism was higher in Asians than in Caucasians. In contrast, the V/L380 polymorphism was higher in Caucasians than in Japanese. Considering the racial and geographic distribution, the S/N167 polymorphism seems to be much older than the V/L380 polymorphism.

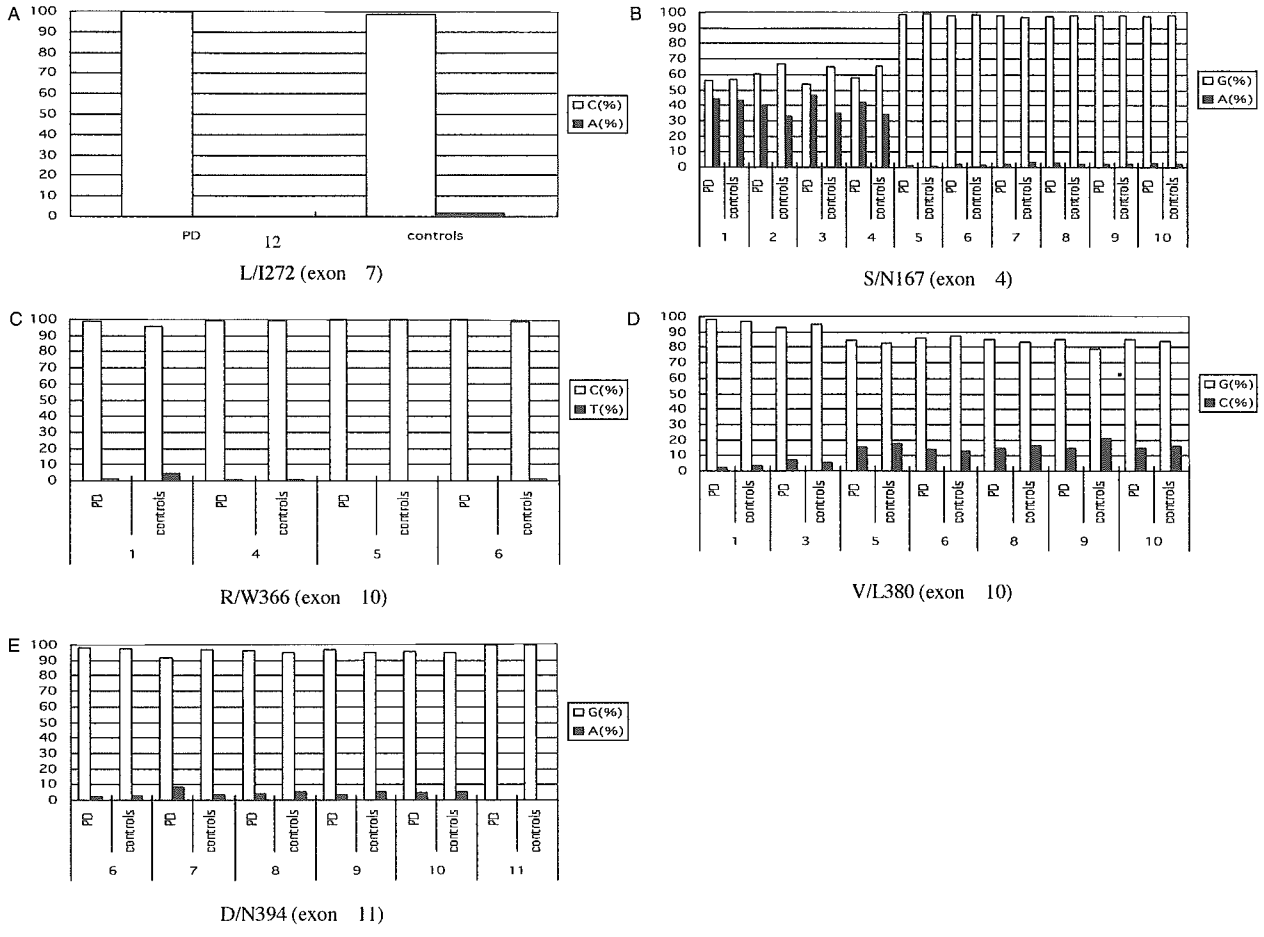

Fig. 3. *Parkin* polymorphisms and frequencies of various alleles in various countries. (A) *Parkin* polymorphism of L/I272; (B) *Parkin* polymorphism of S/N167; (C) *Parkin* polymorphism of R/W366; (D) *Parkin* polymorphism of V/L380; (E) *Parkin* polymorphism of D/N394. 1, Japan (Ref. [13]); 2, Japan (Ref. [12]); 3, China (Ref. [16]); 4, Taiwan (Ref. [17]); 5, Finland (Ref. [18]); 6, Italy (Ref. [20]); 7, Spain (Ref. [19]); 8, North America (Ref. [15]); 9, European1 (Ref. [21]); 10, European2 (Ref. [22]); 11, Japan (this study about D/N394); 12, Japan (this study about L/I272).

Table 2
Allele frequencies of S/N167, R/W366, V/L380 and D/N394 polymorphisms in *parkin* in patients with sporadic PD (SPD) and normal control subjects in different countries

	Japan (Wang)	Japan (Satoh)	China	China (Taiwan)	Finland	Italy	Spain	North America	Europe1	Europe2
S/N167 polymorphism										
Allele G (%)										
SPD	56.6	60.6	53.4	58.2	99.0	97.9	98.0	97.5	97.9	97.5
Control	56.3	66.9	64.9	65.8	99.5	98.5	97.0	98.4	98.0	97.9
Allele A (%)										
SPD	43.4	39.4	46.6	41.8	1.0	2.1	2.0	2.5	2.1	2.5
Control	43.7	33.1	35.1	34.2	0.5	1.5	3.0	1.6	2.0	2.1
Genotype GG (%)										
SPD	36.9	29.6	24.1		98.0	95.8	95.3	94.9	96.1	
Control	36.3	44.0	41.1		99.0	97.0	94.7	96.9	96.0	
Genotype GA (%)										
SPD	39.4	62.0	58.6		2.0	4.2	4.7	5.1	3.9	
Control	40.0	45.9	47.6		1.0	3.0	5.3	3.1	4.0	
Genotype AA (%)										
SPD	23.7	8.4	17.3		0	0	0	0	0	
Control	23.7	10.1	11.3		0	0	0	0	0	
P-value	0.9365	0.23	0.0903	0.1232	0.7111	0.6527	0.8359	0.4532	0.9762	0.5309
R/W366 polymorphism										
Allele C (%)										
SPD	98.8			99.5	100	100				
Control	95.7			99.5	100	99.0				
Allele T (%)										
SPD	1.2			0.5	0	0				
Control	4.3			0.5	0	1.0				
Genotype CC (%)										
SPD	97.5				100	100				
Control	91.3				100	98.0				
Genotype CT (%)										
SPD	2.5				0	0				
Control	8.7				0	2.0				
Genotype TT (%)										
SPD	0				0	0				
Control	0				0	0				
P-value	0.0168			0.9774		0.1670				
V/L380 polymorphism										
Allele G (%)										
SPD	98.1			92.9	85.0	86.3		85.5	85.6	85.1
Control	96.6			94.9	83.0	87.5		83.4	78.8	84.1
Allele C (%)										
SPD	1.9			7.1	15.0	13.7		14.5	14.4	14.9
Control	3.4			5.1	18.0	12.5		16.6	21.2	15.9
Genotype GG (%)										
SPD	96.3				72.1	75.8		72.4	77.5	
Control	93.1				68.6	77.0		75.2	62.4	
Genotype GC (%)										
SPD	3.7				25.8	21.1		25.5	21.6	
Control	6.9				27.7	21.1		21.8	32.8	
Genotype CC (%)										
SPD	0				2.0	3.1		2.1	0.9	
Control	0				3.7	2.0		3.0	4.8	
P-value	0.2190			0.4585	0.4094	0.7288		0.7271	0.0077	0.5198
D/N394 polymorphism										
Allele G (%)										
SPD						98.15	92.0	96.4	96.6	95.3
Control						97.5	97.0	94.9	95.2	95.1

(*continued on next page*)

Table 2 (*continued*)

	Japan (Wang)	Japan (Satoh)	China	China (Taiwan)	Finland	Italy	Spain	North America	Europe1	Europe2
Allele A (%)										
SPD						1.85	8.0	3.6	3.4	4.7
Control						2.5	3.0	5.1	4.8	4.9
Genotype GG (%)										
SPD						93.7	85.9	91.3	93.1	
Control						95.0	95.3	94.4	90.4	
Genotype GA (%)										
SPD						6.3	12.9	7.5	6.9	
Control						5.0	4.7	5.6	9.6	
Genotype AA (%)										
SPD						0	1.2	1.3	0	
Control						0	0	0	0	
P-value						0.1540	0.0029	0.2240	0.4688	0.8604

Japan Wang (Ref. [13]); Japan Satoh (Ref. [12]); China (Ref. [16]); China Taiwan (Ref. [17]); Finland (Ref. [18]); Italy (Ref. [20]); Spain (Ref. [19]); North America (Ref. [15]); Europe1 (Ref. [21]); Europe2 (Ref. [22]).

Table 3
Allele frequencies of V/L380 polymorphism in *parkin* in Asians and Caucasians

	Asian (%)	Caucasians (%)	Total (%)
S/N167 polymorphism			
Number of subjects	568	1335	1903
Number of chromosomes	1136	2670	3806
Allele frequency			
Allele G	646 (56.9%)	2621 (98.2%)	3267 (85.8%)
Allele A	490 (43.1%)	49 (0.8%)	539 (14.2%)
R/W366 polymorphism			
Number of subjects	320	195	515
Number of chromosomes	640	390	1030
Allele frequency			
Allele C	622 (97.2%)	388 (9.5%)	1010 (98.1%)
Allele T	18 (2.8%)	2 (0.5%)	20 (1.9%)
V/L380 polymorphism			
Number of subjects	320	1039	1359
Number of chromosomes	640	2078	2718
Allele frequency			
Allele G	623 (97.3%)	1890 (91.0%)	2513 (92.5%)
Allele C	17 (2.7%)	188 (9.0%)	205 (7.5%)
D/N394 polymorphism			
Number of subjects	400	668	1068
Number of chromosomes	800	1336	2136
Allele frequency			
Allele G	800 (100%)	1275 (95.4%)	2075 (97.1%)
Allele A	0 (0%)	61 (4.6%)	61 (2.9%)

There were significant differences in allele frequencies of S/N167 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 1118.127$, df = 1, $p < 0.0001$). There were significant differences in allele frequencies of R/W366 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 6.731$, df = 1, $p = 0.0095 < 0.01$). There were significant differences in allele frequencies of V/L380 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 28.659$, df = 1, $p < 0.0001$). There were significant differences in allele frequencies of D/N394 polymorphism in *parkin* between Asians and Caucasians ($\chi^2 = 37.601$, df = 1, $p < 0.0001$).

The above results and those of previous studies [10–20] suggest ethnic differences in allele frequencies. It is important to identify the differences in the frequencies among various populations, because single nucleotide polymorphism may not only be involved in the pathogenesis of the disease as a risk or a protective factor, but could also explain the geographic distribution.

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A Combinatorial Code for the Interaction of α-Synuclein with Membranes*

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Considerable genetic and pathological evidence has implicated the small, soluble protein α-synuclein in the pathogenesis of familial and sporadic forms of Parkinsons disease (PD). However, the precise role of α-synuclein in the disease process as well as its normal function remain poorly understood. We recently found that an interaction with lipid rafts is crucial for the normal, pre-synaptic localization of α-synuclein. To understand how α-synuclein interacts with lipid rafts, we have now developed an *in vitro* binding assay to rafts purified from native membranes. Recapitulating the specificity observed *in vivo*, recombinant wild type but not PD-associated A30P mutant α-synuclein binds to lipid rafts isolated from cultured cells and purified synaptic vesicles. Proteolytic digestion of the rafts does not disrupt the binding of α-synuclein, indicating an interaction with lipid rather than protein components of these membranes. We have also found that α-synuclein binds directly to artificial membranes whose lipid composition mimics that of lipid rafts. The binding of α-synuclein to these raft-like liposomes requires acidic phospholipids, with a preference for phosphatidylserine (PS). Interestingly, a variety of synthetic PS with defined acyl chains do not support binding when used individually. Rather, the interaction with α-synuclein requires a combination of PS with oleic (18:1) and polyunsaturated (either 20:4 or 22:6) fatty acyl chains, suggesting a role for phase separation within the membrane. Furthermore, α-synuclein binds with higher affinity to artificial membranes with the PS head group on the polyunsaturated fatty acyl chain rather than on the oleoyl side chain, indicating a stringent combinatorial code for the interaction of α-synuclein with membranes.

Recent work has indicated an important role for the protein α-synuclein in the pathogenesis of Parkinsons disease (PD).[1] Mutations in α-synuclein produce a highly penetrant but rare autosomal dominant form of PD (1–3). In other families, increased dosage of the wild type gene suffices to cause PD (4). Although mutations in α-synuclein do not contribute to idiopathic PD, the brains of most patients contain abundant α-synuclein in the form of Lewy bodies and dystrophic neurites (5–7), supporting a role for the protein in sporadic forms of the disease. However, the mechanism by which α-synuclein contributes to neural degeneration remains poorly understood.

Originally identified as a synaptic vesicle-associated protein, α-synuclein has been implicated in synaptic plasticity, neurotransmitter release, and more specifically, synaptic vesicle recycling (8–12). Despite its specific localization to the nerve terminal, α-synuclein does not co-fractionate with native membranes in brain extracts, but behaves as a soluble protein (13–16). The molecular determinants that localize α-synuclein to the synapse thus remain unknown. However, α-synuclein can associate with native membranes such as axonal transport vesicles, lipid droplets produced in HeLa cells by the administration of oleic acid, and the membranes of *Saccharomyces cerevisiae* (17–19). Importantly, the A30P mutation associated with familial PD disrupts these interactions. *In vitro*, α-synuclein binds directly to artificial membranes containing acidic phospholipids in a manner that is not sensitive to the A30P mutation (20–23). The relationship of these observations to the pre-synaptic localization of α-synuclein has remained unclear.

Recently, we found that α-synuclein associates specifically with membrane microdomains known as lipid rafts (24). Lipid rafts are enriched in cholesterol, sphingomyelin, and phospholipids with saturated long chain fatty acids as well as particular proteins. Biochemically, they are defined by their insolubility in cold Triton X-100 and their low buoyant density (25). α-Synuclein expressed in cultured cells and in brain co-fractionates with detergent-resistant membranes, consistent with its raft association. Pharmacologic disruption of lipid rafts eliminates the synaptic enrichment of α-synuclein. The A30P mutation associated with familial PD also disrupts the raft association of α-synuclein, and redistributes the protein from synapses into the axon (24). Binding to lipid rafts thus contributes to the normal function of α-synuclein by localizing the protein to the nerve terminal, and may also influence its role in the pathogenesis of PD.

To define the interaction of α-synuclein with lipid rafts, we

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[1] The abbreviations used are: PD, Parkinsons disease; PS, phosphatidylserine; GST, glutathione *S*-transferase; PC, phosphatidylcholine; MES, 4-morpholineethanesulfonic acid; BB, binding buffer; DRM, detergent-resistant membrane; PK, proteinase K; CBP, calmodulin-binding peptide; SM, sphingomyelin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

have developed an *in vitro* assay for binding to membranes prepared from native tissue. Using this assay, recombinant wild type α-synuclein binds saturably and with high affinity to lipid rafts isolated from HeLa cells and rat brain, and the A30P mutation disrupts the interaction. The assay thus faithfully recapitulates the specificity of raft association observed in cells (24). Proteolytic digestion of the raft fraction does not reduce raft association *in vitro*, indicating a direct interaction of α-synuclein with the membrane. Confirming a direct lipid interaction, α-synuclein binds to artificial membranes mimicking the composition of lipid rafts (25). Consistent with previous reports (20, 26, 27), the interaction of α-synuclein with raft-like liposomes requires phospholipids with an acidic head group, with a strong preference for phosphatidylserine (PS). In contrast to previous studies, however, our assay reveals specific requirements for the acyl chain composition of PS. A variety of synthetic PS with single defined acyl chains do not support binding of α-synuclein. Rather, binding requires a combination of PS with oleic (18:1) and polyunsaturated (20:4 and 22:6) fatty acyl chains, suggesting that a phase transition contributes to the association with lipid rafts. Furthermore, α-synuclein binds preferentially to membranes with PS on the polyunsaturated acyl chain, indicating the coordinate recognition of head group in the context of a specific side chain.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to human α-synuclein (15G7), rat α-synuclein (Syn1), VGLUT1, GST, and CD55 were obtained from, respectively, Alexis Biochemicals (San Diego, CA), BD Biosciences (San Diego, CA), Chemicon (Temecula, CA), Molecular Probes (Eugene, OR), and Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Amersham Biosciences. Secondary antibodies conjugated to fluorescein isothiocyanate or Cy3 and Alexa 647-conjugated Annexin V were obtained from Jackson ImmunoResearch (West Grove, PA) and Molecular Probes (Eugene, OR), respectively. Cholesterol, brain sphingomyelin (SM), brain PS, egg phosphatidic acid, brain phosphatidylcholine (PC), brain phosphatidylethanolamine, 1,2-distearoyl-*sn*-glycero-3-phosphoserine (18:0 PS), 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (18:1 PS), 1,2-diarachidonoyl-*sn*-glycero-3-phosphoserine (20:4 PS), and 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphoserine (22:6 PS) were obtained from Avanti Polar Lipids (Alabaster, AL).

Molecular Biology and Cell Culture—The construction of α-synuclein cDNAs and purification of bacterial fusion proteins has been described previously (24). HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% cosmic calf serum (HyClone, Logan, UT) at 37 °C and 5% CO_2. Dissociated hippocampal cultures containing glia were prepared from embryonic (E18.5) rats and maintained in Neurobasal medium (Invitrogen, San Diego, CA) for 2–3 weeks (28).

Preparation of Synaptic Vesicles from Rat Brain—Synaptic vesicles were prepared as previously described (29, 30). Briefly, the cortices of 200-g male Sprague-Dawley rats were homogenized in 0.32 M sucrose, 4 mM HEPES-NaOH, pH 7.4, 1 mM NaF, 1 mM Na_3VO_4, 10 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, containing phosphatase inhibitor mixtures I and II (Calbiochem, La Jolla, CA) (HB). Cell debris was removed by centrifugation at 1,350 × *g* for 10 min at 4 °C, and crude synaptosomes were sedimented at 12,000 × *g* for 10 min at 4 °C. The synaptosomal pellet was washed in HB, sedimented at 13,000 × *g* for 15 min, and the resulting pellet lysed by hypo-osmotic shock in ice-cold water containing protease and phosphatase inhibitors. Lysed synaptosomes were adjusted to 9.3 mM HEPES, pH 7.4, and synaptic plasma membrane removed by centrifugation at 33,000 × *g* for 20 min. The resulting supernatant was further sedimented at 260,000 × *g* for 2 h to pellet synaptic vesicles, which were resuspended in 25 mM MES, 80 mM NaCl (binding buffer, BB) containing protease and phosphatase inhibitors as above.

Isolation of Detergent-resistant Membranes (DRMs)—DRMs were isolated from HeLa cells and synaptic vesicles as previously described (24, 31). Briefly, HeLa cells (1×10^7) or synaptic vesicles (200 μg of protein) were resuspended in 1 ml of BB containing 1% Triton X-100 and incubated on ice for 30 min with Dounce homogenization every 10 min. The resulting extract was adjusted to 42.5% sucrose, overlaid with 5 ml each of 35 and 5% sucrose in BB, and sedimented at 4 °C in a Beckman SW41 rotor at 275,000 × *g* for 18 h. Lipid rafts (250 μl) were collected at the interface between 5 and 35% sucrose, and stored at 4 °C until use.

Proteinase K (PK) Digestion—100 μl of isolated DRMs were dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and incubated with 10 μl of PK-agarose (Sigma) at 30 °C with rotation for the times indicated. The reaction was terminated by sedimentation of the PK-agarose. PK-treated rafts were subjected to electrophoresis through Criterion Tris-HCl polyacrylamide gels (Bio-Rad) followed by staining with the GelCode SilverSNAP Stain Kit (Pierce).

Preparation of Liposomes—Lipids of interest were mixed in chloroform, and the solvent evaporated under nitrogen. The resulting lipid film was dried under vacuum for 20 min and re-hydrated at a concentration of 2.5 mM in BB followed by vortexing. Small unilamellar vesicles were prepared by five 1-min sonication and freeze/thawing cycles, stored in the dark at 4 °C under nitrogen, and used within 1 week of production.

In Vitro Binding Assay—Recombinant α-synuclein, fused at its N terminus to the 41-residue calmodulin-binding peptide (CBP), was combined with 100 μl of DRMs or 5 μl of liposomes in BB containing 1% bovine serum albumin as nonspecific competitor, and incubated at 30 °C for 30 min. Sucrose was then added to a final concentration of 42.5%, the mixture was overlaid with 2 ml of 35% and 2 ml of 5% sucrose in BB, and sedimented at 4 °C in a Beckman SW55 rotor at 275,000 × *g* for 18 h. Ten 0.5-ml fractions were collected from the top of the gradient and either used immediately or stored at −80 °C until use, with no differences observed between unfrozen and frozen material (data not shown). Equal volumes of each fraction were separated by electrophoresis as above, electrotransferred to polyvinylidene difluoride, immunostained with appropriate antibodies, and detected using West Pico SuperSignal (Pierce). For quantitative Western blotting, protein bands were quantified using the ChemiImager System (Alpha Innotech, San Leandro, CA). Every experiment was performed independently at least twice. Unless indicated otherwise, all experiments involved the CBP fusion to α-synuclein.

Purification of Bound α-Synuclein for Mass Spectrometry—Binding was performed as above using a final concentration of 1 mM liposomes and 8 μM α-synuclein. Membrane-associated α-synuclein was collected from the 5/35% sucrose interface, solubilized in 20 mM CHAPS, the micelles were removed by centrifugation through a 10-kDa molecular mass cutoff Amicon Ultra filter (Millipore, Bedford, MA), and the buffer exchanged to 100 mM ammonium bicarbonate. For further purification, concentrated α-synuclein was separated by size exclusion chromatography on a Superose 12 column (Amersham Biosciences) in 100 mM ammonium bicarbonate at a flow rate of 0.2 ml/min. Fractions containing α-synuclein were identified by Western blotting and dried under vacuum centrifugation. Each sample was then digested overnight with 100 ng of trypsin (sequencing grade modified, Promega, Madison, WI) in 25 mM ammonium bicarbonate. Samples were analyzed by liquid chromatography-mass spectrometry using a nano-LC system (Eksigent, Livermore, CA) to separate samples for on-line analysis using a QSTAR mass spectrometer (Sciex, Concord, Ontario, Canada). The resulting data were analyzed using a combination of manual analysis and automated analysis with the locally developed Protein Prospector suite of proteomic tools (www.prospector.ucsf.edu).

Immunofluorescence—Hippocampal neurons were grown for 15–20 days *in vitro*, fixed in 4% paraformaldehyde, and immunostained for α-synuclein and VGLUT1. When indicated, Alexa 647-conjugated Annexin V was added in 2.5 mM $CaCl_2$ during the last 15 min of incubation with secondary antibodies. Fluorescent images were acquired on a Zeiss (Oberkochen, Germany) LSM 510 confocal microscope. Annexin V was pre-adsorbed by incubation with liposomes overnight at 4 °C, the bound material was removed by sedimentation at 100,000 × *g*, and the supernatant used for immunofluorescence. To allow direct comparison of Annexin V staining, images were collected using fixed laser strength, pinhole size, and detector gain.

RESULTS

α-Synuclein Binds to Purified Lipid Rafts—To understand how α-synuclein interacts with lipid rafts, we have developed an *in vitro* binding assay using recombinant α-synuclein and DRMs prepared from HeLa cells by flotation gradient (24, 31). The DRMs were incubated with recombinant α-synuclein for 30 min at 30 °C, and bound protein was separated by flotation through a second density gradient. Initially, we observed binding of both wild type and A30P α-synuclein to purified DRMs

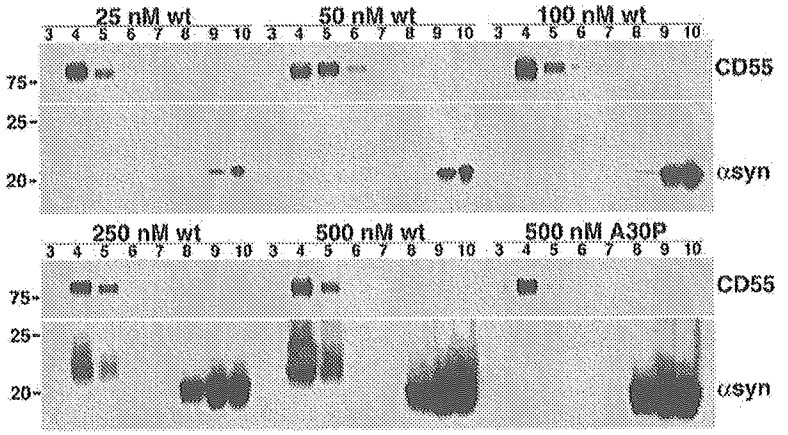

FIG. 1. **α-Synuclein binds to purified DRMs and the A30P mutation associated with PD disrupts the interaction.** Increasing concentrations of recombinant human α-synuclein (*wt*) (25–500 nM CBP fusion) were incubated at 30 °C for 30 min with DRMs purified from HeLa cells in the presence of bovine serum albumin (1%) as nonspecific competitor. Bound protein was separated from free by flotation on a sucrose density gradient, and the resulting fractions immunoblotted for α-synuclein. *Numbers above* the gels indicate fractions starting at the top of the gradient. Immunoblotting for the glycosylphosphatidylinositol-anchored protein CD55 identifies the rafts in fractions 4–6. Increasing amounts of α-synuclein result in increased binding, whereas the A30P mutant shows no binding even at the highest concentration tested (500 nM). DRM-bound α-synuclein also exhibits a shift in gel mobility similar to that previously observed (24). *Numbers* on the *left* indicate size markers (in kDa).

(data not shown), very similar to the results of other *in vitro* binding studies (20–23) but different from the lack of membrane association observed for the A30P mutant *in vivo* (17–19, 24). Supporting the possibility that A30P α-synuclein might interact nonspecifically with rafts under these conditions, we have observed substantial adsorption of α-synuclein to a variety of surfaces including plastic as well as membranes (data not shown). However, the high protein concentrations present in the cytoplasm of cells presumably reduce these nonspecific interactions *in vivo*. To test this possibility, we added BSA to the *in vitro* binding assay. Under these conditions, a substantial proportion of wild type α-synuclein (15% at 250 nM and ~25% at 500 nM) co-migrates in light fractions with CD55, a glycosylphosphatidylinositol-anchored protein of lipid rafts, indicating association with DRMs (Fig. 1). In contrast, A30P-α-synuclein does not bind to DRMs in the presence of BSA, even at the highest concentration of recombinant protein used in this study (Fig. 1). The association of wild type α-synuclein with DRMs is dose-dependent, with binding detectable in the mid-nanomolar range. We also found that varying the salt (NaCl or KCl), salt concentration (from 50 to 110 mM), or pH (from 6.5 to 8) has no detectable effect on the binding of α-synuclein to DRMs (data not shown). Because α-synuclein depends on lipid rafts for its specific localization to the nerve terminal, we examined binding to DRMs prepared from synaptic vesicles, and observed similar high affinity binding and sensitivity to the A30P mutation (data not shown). Because the recombinant protein used for these studies contains a 41-residue CBP fused to the N terminus of α-synuclein, we also removed the CBP tag by proteolytic cleavage with enterokinase (leaving no additional amino acids), and observed the same high affinity, specific binding to DRMs from HeLa cells and synaptic vesicles (data not shown). α-Synuclein bound to DRMs *in vitro* migrates more slowly by gel electrophoresis than soluble α-synuclein at the bottom of the flotation gradient (Fig. 1), indicating the acquisition *in vitro* of a raft-associated modification very similar to that observed *in vivo* (24). The *in vitro* binding assay therefore recapitulates many features observed for the interaction of α-synuclein with rafts in cells.

We used a competition assay to characterize further the association of α-synuclein with rafts. In particular, we assessed the ability of CBP-α-synuclein to compete with GST-α-synuclein for binding to DRMs. Fig. 2*A* shows that increasing amounts of wild type CBP-α-synuclein inhibit binding of 100 nM wild type GST-α-synuclein, indicating that the interaction with membranes is saturable. Quantification of multiple experiments reveals that concentrations of CBP-α-synuclein, ~0.5 μM, essentially eliminate binding of GST-α-synuclein (Fig. 2*B*), indicating an affinity that is at least in the mid-nanomolar range. Importantly, 2 μM A30P-CBP-α-synuclein fails to eliminate binding of wild type GST-α-synuclein (Fig. 2), confirming that this PD-associated mutant binds to DRMs with lower affinity than wild type (24).

Requirement for the Lipid Component of DRMs—To determine whether α-synuclein interacts with the protein or lipid component of rafts, we treated DRMs isolated from HeLa cells with proteinase K (PK) immobilized on agarose beads. After digestion for 5–60 min, PK-agarose was removed by sedimentation before adding recombinant α-synuclein. Strikingly, proteolytic digestion with PK did not reduce the interaction of α-synuclein with DRMs (Fig. 3*A*). However, a substantial proportion of CD55 remains intact even after prolonged incubation in proteinase K (Fig. 3*A*), raising questions about the extent of digestion. Resistance to proteolysis could simply reflect the presence of CD55 on the lumenal face of isolated DRMs, inaccessible to PK. Lumenal proteins presumably do not participate in the interaction with recombinant α-synuclein. We further confirmed that digestion in PK for 60 min largely eliminates DRM protein as detected by silver staining (Fig. 3*B*). In similar experiments using DRMs isolated from synaptic vesicles, treatment with PK also fails to inhibit binding of α-synuclein (data not shown). Remarkably, the α-synuclein bound to PK-digested DRMs still shows retarded electrophoretic mobility (Fig. 3*A*), indicating that this modification is unlikely to require proteins such as the raft-enriched kinases known to phosphorylate α-synuclein (32–34).

α-Synuclein Binds to Artificial Liposomes Resembling Lipid Rafts—To test further the hypothesis that α-synuclein associates with rafts through lipid rather than protein interactions, we prepared artificial membranes that resemble lipid rafts. Specifically, we used the "canonical" raft mixture composed of cholesterol, brain sphingomyelin (SM), and brain phospholipid (1:1:1 molar ratio), which mimics the enrichment of cholesterol and SM observed in native rafts (25). α-Synuclein binds to these liposomes and exhibits the same gel mobility shift observed when α-synuclein associates with native lipid rafts (Fig.

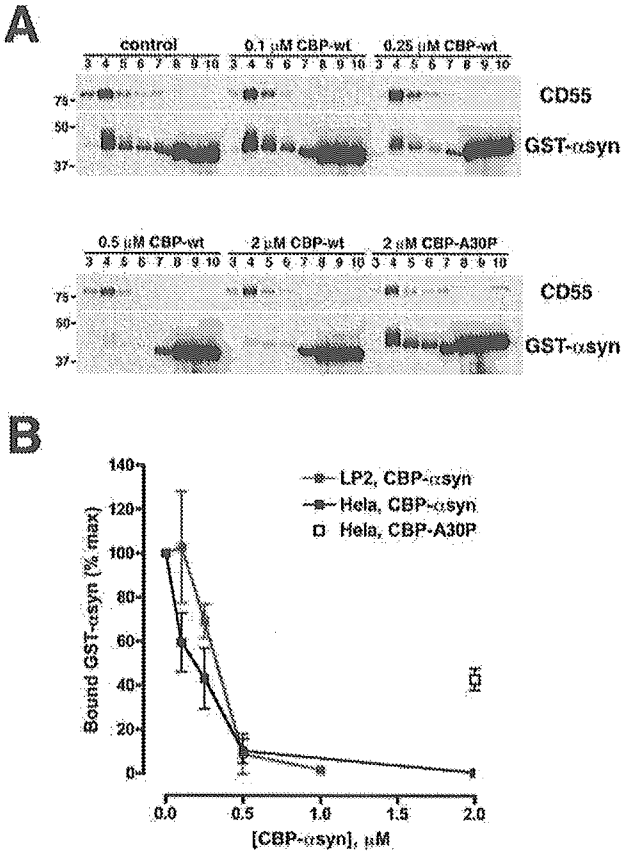

FIG. 2. **CBP-α-synuclein competes with GST-α-synuclein for binding to DRMs.** Wild type (*wt*) GST-α-synuclein (0.1 μM) and either wild type CBP-α-synuclein (0.1–2 μM) or CBP-A30P-α-synuclein (2 μM) were incubated with DRMs purified from HeLa cells or synaptic vesicles (LP2), and the raft-bound protein were separated by flotation through a sucrose density gradient as described in the legend to Fig. 1.*A*, fractions were immunoblotted for GST and in the case of HeLa DRMs, CD55. Increasing concentrations of wild type CBP-α-synuclein block the binding of wild type GST-α-synuclein to DRMs. In contrast, A30P CBP-α-synuclein reduces the binding of wild type protein only at high concentrations, confirming that the mutant has a lower affinity for DRMs than wild type α-synuclein. *Numbers* on the *left* indicate size markers (in kDa). *B*, the chemiluminescent output of Western blots was quantified by collecting the emitted light and the resulting signal normalized to the maximal signal obtained in the absence of competitor CBP fusion protein. The *graph* shows the mean of at least three independent experiments, and the *error bar* indicates S.D.

4*A*). Consistent with the results obtained from PK-digested DRMs, lipids alone thus support the binding of α-synuclein.

We varied the composition of the artificial membranes to identify the components of lipid rafts recognized by α-synuclein. Removal of cholesterol or SM (with replacement by brain PC) does not reduce the binding of α-synuclein to raft-like liposomes (Fig. 4*B*), indicating that the protein does not directly interact with these lipids. Indeed, a reduction in cholesterol content increases the binding of α-synuclein. The well described disruption of raft integrity by cholesterol depletion, rather than the loss of a direct interaction with α-synuclein, must therefore account for the effects of cholesterol depletion *in vivo* (24). In addition, it has been reported that the canonical raft mixture resides close to the miscibility transition temperature where small changes in vesicle composition have large effects on phase behavior (35, 36). The increased binding observed with decreased cholesterol may thus reflect an increase in the number of raft domains.

Acidic phospholipids promote the binding of α-synuclein to raft-like liposomes (Fig. 4*C*). PS is particularly effective, but phosphatidic acid also supports the interaction. In contrast, neutral phospholipids, such as PC and phosphatidylethanolamine, do not support the binding of α-synuclein to raft-like liposomes, consistent with previous reports using non-raft liposomes (20, 37). It is believed that lysines present on the α-helix induced by membrane association interact directly with the negatively charged phospholipid head group (20, 21, 23). However, other observations suggest that head group cannot be the only feature of raft phospholipid required for the membrane association of α-synuclein.

α-Synuclein Colocalizes with a Subset of Membrane PS—If PS confers the specific localization of α-synuclein to the synapse, then PS should exhibit a similar synaptic enrichment. To determine the distribution of PS, we have taken advantage of its specific recognition by annexin V (38). Most often used in live cells as a marker for the appearance of PS on the outer leaflet of the plasma membrane during apoptosis, annexin V has also been used in fixed cells to label intracellular compartments (39). Indeed, we observed extensive calcium-dependent labeling of primary hippocampal neurons with annexin V (Fig. 5*A*). To test further the specificity for PS, we adsorbed the annexin V with liposomes containing either PS and PC (1:1) or PC alone. Adsorption with membranes containing PS completely eliminated the labeling, whereas membranes containing PC alone had no effect, confirming the specificity of staining with annexin V. The extensive labeling of cultured neurons with annexin V thus reflects the true distribution of PS, which co-localizes only in part with the labeling for α-synuclein and the synaptic vesicle glutamate transporter VGLUT1 (Fig. 5*B*). Additional determinants must therefore account for the specific localization of α-synuclein to the nerve terminal.

Role of the Acyl Chain—α-Synuclein has been reported to bind monounsaturated free fatty acids such as oleic acid (18:1) and to form multimers in the presence of polyunsaturated fatty acids such as arachidonic acid (20:4) and docosahexaenoic acid (22:6) (40, 41). However, the role of phospholipid acyl chains in membrane binding of α-synuclein has not been addressed directly. We therefore varied the acyl chain composition of PS in liposomes containing cholesterol, sphingomyelin (SM), and PS (1:1:1). Because the brain PS used for these studies contains a mixture of acyl side chains (1.1% 16:0, 41.8% 18:0, 33.7% 18:1, 2.4% 20:4, 8.4% 22:6, 12.6% other), we first prepared membranes containing individual, defined synthetic forms of PS with both acyl chains identical (symmetric). However, none of the major components of brain PS (18:0, 18:1, 20:4, and 22:6) supports the interaction with α-synuclein when used alone (Fig. 6*A*). With large amounts of lipid and recombinant protein (8 times higher than the standard assay described above), we detected binding of α-synuclein to raft-like liposomes containing 18:1 PS alone (with cholesterol and SM) (Fig. 6*B*). Under these conditions, however, α-synuclein does not undergo the shift in gel mobility observed with rafts prepared from native tissue or with raft-like liposomes containing brain PS (Fig. 6*B*). We took advantage of this phenomenon to determine whether the altered mobility reflects a covalent modification. Analysis of trypsin-digested "shifted" α-synuclein (bound to membranes containing brain PS) and "unshifted" protein (bound to membranes with 18:1 PS) by mass spectrometry identified peptides spanning all of the α-synuclein sequence except the first 10 amino acids (MDVFMKGLSK) (data not shown). All peptides were detected only in an unmodified state, ruling out any stoichiometric covalent modifications of this part of the protein. Because we did not identify peptides spanning the first 10 residues of α-synuclein, we cannot exclude their potential modification. However, despite there being multiple studies dem-

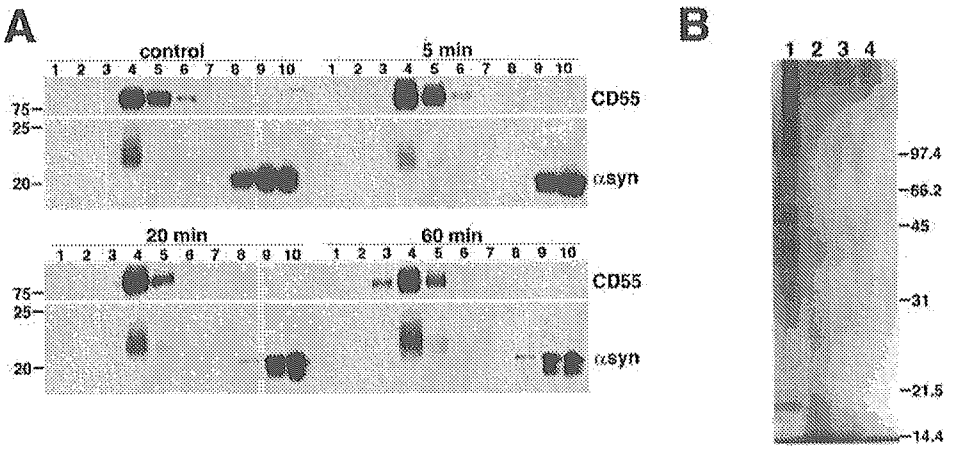

FIG. 3. **Binding of α-synuclein to DRMs does not require protein.** HeLa DRMs pretreated with PK for 5–60 min at 30 °C were incubated with recombinant human α-synuclein (50 nM CBP fusion) and subjected to flotation through a sucrose gradient as described above. Raft fractions are identified as CD55-positive. *A*, Western analysis of α-synuclein shows no effect of PK digestion on the raft association of α-synuclein. In addition, digestion with PK does not affect the shift in gel mobility of raft-bound α-synuclein. *B*, silver staining of raft extracts treated with PK for 0, 5, 20, and 60 min (*lanes 1–4*) shows a progressive loss of detectable raft protein. *Numbers* on the *left* indicate size markers (in kDa).

onstrating the post-translational modification of α-synuclein, none have reported modifications on any of the first 10 residues of the protein (32–34, 42–45). Together with the appearance of the gel mobility shift after binding to artificial membranes with no additional protein, the shift seems very unlikely to reflect a covalent modification.

Because no individual form of synthetic PS alone confers binding to α-synuclein, we combined PS with defined acyl chains in proportions mimicking those found in brain PS. Very similar to brain PS, this combination supports binding (Fig. 7*A*, *upper row*, *middle panel*). To determine the specific acyl chain requirements, we examined various mixtures of synthetic PS, focusing on simpler combinations with PS containing saturated or monounsaturated acyl chains and polyunsaturated side chains. To mimic the physiological proportions found in brain PS, we used more of the saturated or monounsaturated PS, and less of the polyunsaturated PS. Fig. 7*A* (*lower row*, *middle panel*) shows that 18:0 PS with either 20:4 or 22:6 PS exhibits weak binding. In contrast, 18:1 PS confers robust binding when combined with 20:4 or 22:6 PS (Fig. 7*A*, *lower row*, *left* and *right panels*), similar to that seen with brain PS and the reconstituted mixture of synthetic PS. α-Synuclein thus specifically requires both 18:1 PS and PS containing polyunsaturated acyl chains for its interaction with raft-like liposomes.

We further determined the proportion of 18:1 and 20:4 PS optimal for the interaction of α-synuclein with raft-like liposomes. Fig. 7*B* (*upper row*, *right panel*) shows that an equimolar combination of 18:1 and 20:4 PS (17% each) confers binding to raft-like liposomes. A 4-fold excess of 18:1 PS over 20:4 PS also promotes the binding of α-synuclein (Fig. 7*B*, *lower row*, *left panel*), but an excess of 20:4 PS over 18:1 PS (*upper row*, *middle panel*) does not. Further increases in the proportion of either 18:1 or 20:4 PS, with proportionate decreases of the other, completely abolish association of α-synuclein with the liposomes (Fig. 7*B*). Because 18:1 PS predominates over polyunsaturated PS in native membranes, α-synuclein apparently recognizes a physiologically relevant combination of fatty acid side chains.

Role of Phase Transition—Although lipid rafts have been shown to contain polyunsaturated fatty acids (46), they are generally thought to be enriched in long chain saturated acyl chains (47). The mechanism by which polyunsaturated phospholipid promotes the binding of α-synuclein to lipid rafts thus remains uncertain. α-Synuclein may interact with the PS containing polyunsaturated acyl chains inside raft domains. Alternatively, polyunsaturated acyl chains may promote a phase transition between raft and non-raft membranes. Indeed, the requirement of α-synuclein binding for both 18:1 and 20:4 PS supports a role for phase transition (Fig. 7). If phase transition is important for membrane association, the addition of any lipid that induces phase transition, including a lipid incapable by itself of binding α-synuclein, should support binding provided that at least some PS is present. We therefore supplemented the cholesterol/SM/18:1 PS liposomes, which cannot alone bind α-synuclein (Fig. 6*A*) with 20:4 PC, which also cannot by itself bind. The addition of 20:4 PC leads to a remarkable increase in the binding of α-synuclein (Fig. 8, *middle row*, *left panel*). Similarly, liposomes containing 18:1 PC and 20:4 PS, which fulfill both the head group and phase transition requirements, also support the binding of α-synuclein (Fig. 8, *middle row*, *middle panel*). However, membranes with the polyunsaturated acyl chain on the PS head group seem to bind with higher affinity than those with the monounsaturated acyl chain on PS within the context of brain PC (Fig. 8, *bottom row*). In addition to a requirement for phase transition, α-synuclein thus binds preferentially to PS containing polyunsaturated acyl chains.

DISCUSSION

To understand how the interaction with lipid rafts localizes α-synuclein to the nerve terminal, we have developed an *in vitro* assay for membrane binding by α-synuclein that recapitulates many features of the interaction observed *in vivo* (24). Using this assay, we show that α-synuclein binds saturably and with high affinity to DRMs isolated from HeLa cells or synaptic vesicles. The interaction is resistant to digestion of the rafts with proteinase K, suggesting a requirement for lipid and not protein. Confirming a direct interaction with lipid, α-synuclein binds to artificial liposomes that mimic rafts. Although other groups have previously shown that α-synuclein binds to liposomes *in vitro* (20–23), the assay described here differs in its sensitivity to the PD-associated mutation A30P. The A30P mutation disrupts the membrane interactions of α-synuclein in cells (17–19, 24), but has had little effect on binding to artificial membranes *in vitro* (20–23). Sensitivity to the A30P mutation thus supports the physiological relevance of this assay and of raft binding. The specificity of the assay presumably reflects the use of nonspecific competitor protein as

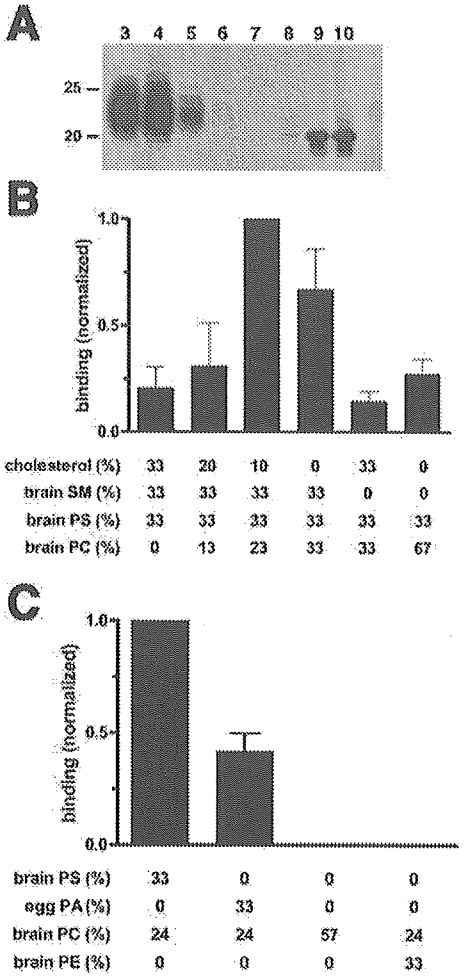

FIG. 4. **α-Synuclein binds to artificial membranes mimicking lipid rafts.** *A*, recombinant α-synuclein (100 nM CBP fusion) was incubated for 30 min at 30 °C with 25 μM artificial membranes containing cholesterol:brain sphingomyelin:brain PS (1:1:1 molar ratio), the bound protein was separated as described in the legend to Fig. 1, and the fractions immunoblotted for α-synuclein. α-Synuclein binds to these artificial membranes, and the bound protein exhibits a shift in gel mobility similar to that observed with rafts prepared from native tissue. *B*, the lipid composition of artificial membranes was modified by substituting cholesterol, brain SM, or brain PS with brain PC. After the binding assay and flotation gradient, the fractions were immunoblotted for human α-synuclein, the immunoreactivity was quantified and expressed as the ratio of bound (fractions 3–5) to unbound (fraction 9–10) α-synuclein, with normalization of the ratios to the maximal binding observed with cholesterol:SM:brain PS:brain PC (10:33:33:23). The results shown are the mean of three independent experiments ± S.D. *C*, α-synuclein (100 nM CBP fusion) was incubated with 25 μM artificial membranes prepared from 10% cholesterol, 33% brain SM, 24% brain PC, and various phospholipids. The binding assay, flotation gradient, and Western analysis were performed as in *A*. α-Synuclein binds only to lipids containing either the acidic PS or phosphatidic acid (*PA*), but not the neutral PC or phosphatidylethanolamine (*PE*). Data shown are the mean of three experiments ± S.D.

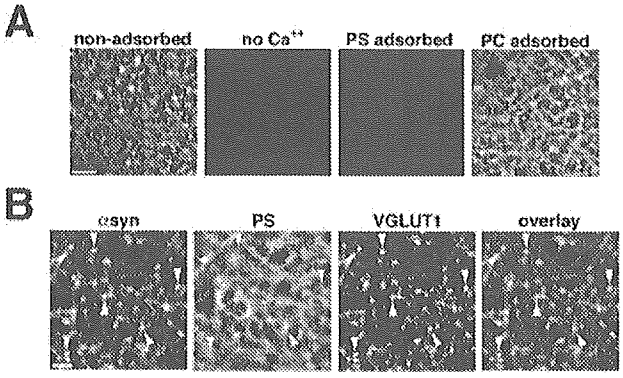

FIG. 5. **α-Synuclein co-localizes with a small subset of neuronal PS.** *A*, fixed hippocampal neurons were stained for PS using annexin V-Alexa 647 (non-adsorbed). The annexin V conjugate binds to all neuronal processes without enrichment in any particular compartment. Removal of calcium (no Ca^{2+}) and adsorption with liposomes containing PC:PS (1:1; PS adsorbed) but not PC alone (PC adsorbed) abolishes labeling with annexin V, confirming the specificity for PS. *Size bar*, 10 μm. *B*, α-synuclein co-localizes with only a subset of PS at synaptic sites that also label for synaptic vesicle glutamate transporter VGLUT1. α-Synuclein thus requires more than the PS head group for localization to the nerve terminal. *Arrowheads* point to synapses labeling for α-synuclein (*green* in merged image), PS (*blue* in merged image), and VGLUT1 (*red* in merged image). *Size bar*, 2 μm.

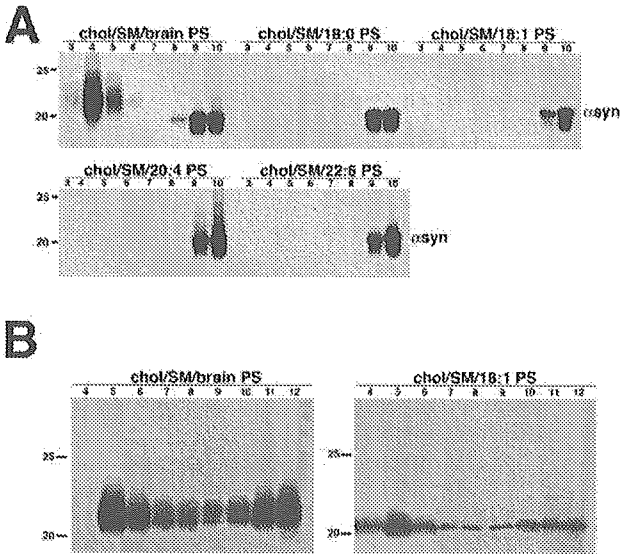

FIG. 6. **α-Synuclein does not bind to raft-like artificial membranes containing synthetic PS with a single defined fatty acyl chain.** *A*, α-synuclein (100 nM CBP fusion) was incubated with 25 μM artificial membranes containing equimolar ratios of cholesterol (*chol*), brain SM, and the indicated brain or synthetic PS. Binding, separation, and Western analysis were performed as described in previous figures. α-Synuclein (*α-syn*) binds strongly only to liposomes containing brain PS. *B*, α-synuclein interacts with raft-like liposomes containing 18:1 PS when protein and lipid are present at higher concentrations (here 8 μM α-synuclein and 1 mM lipid). Under these conditions, the gel mobility of α-synuclein (including soluble protein at the bottom of the gradient as well as membrane-bound protein in higher fractions) is retarded when interacting with brain PS (*left*), but not when interacting with 18:1 PS (*right*). *Numbers* on the *left* indicate size markers (kDa).

well as the raft-like nature of the membranes.

We previously observed that the α-synuclein associated with lipid rafts exhibits a shift in gel mobility (24). α-Synuclein indeed undergoes a conformational change from unstructured to α helical on binding to a variety of membranes *in vitro* (20, 23, 37, 48). However, other studies do not report an alteration in electrophoretic mobility of α-synuclein upon membrane binding. In general, these studies have used artificial membranes containing only synthetic phospholipid, suggesting that native membranes contain an activity missing from artificial membranes, which is required for the modification. The phosphorylation of α-synuclein by tyrosine kinases associated with lipid rafts raised the possibility that the observed gel shift reflects a post-translational modification (32–34, 44). However, extensive proteolysis of purified lipid rafts with proteinase K does not reduce either the binding or gel shift of α-synuclein.

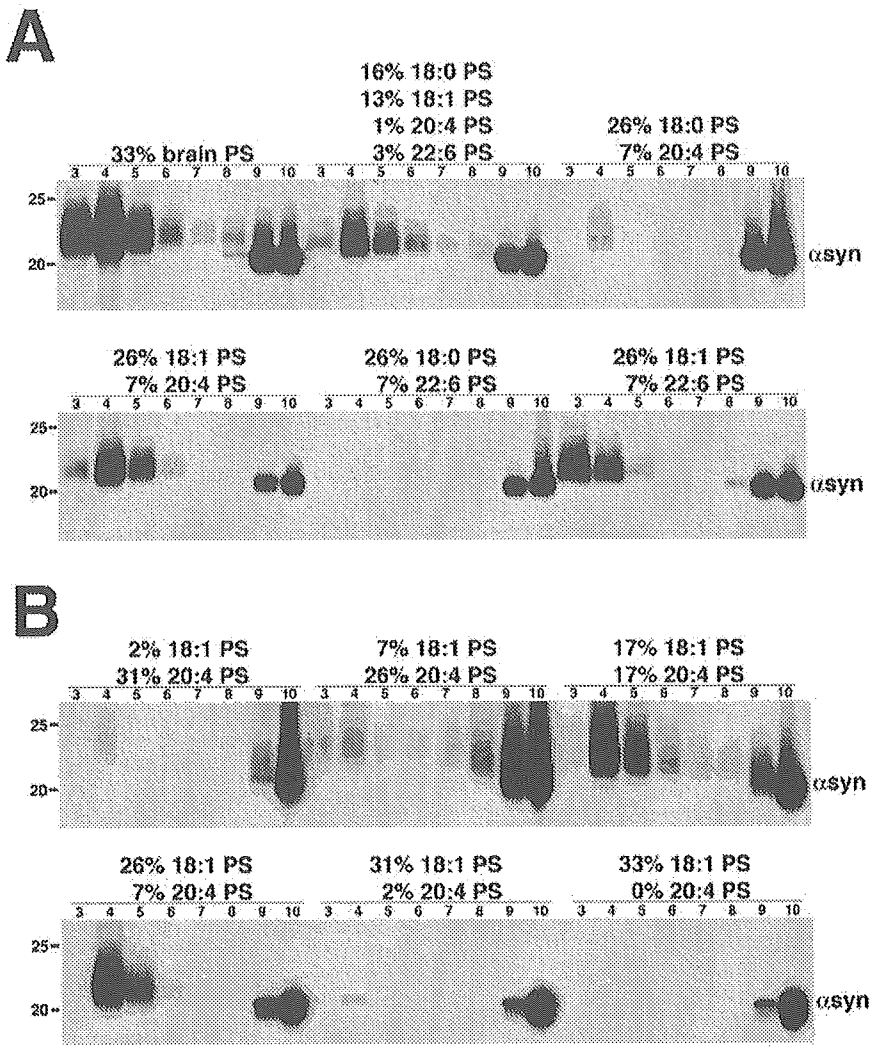

FIG. 7. **α-Synuclein binding requires PS with both mono- and polyunsaturated fatty acyl chains.** *A*, α-synuclein (*α-syn*) (100 nM CBP fusion) was incubated with liposomes containing equimolar concentrations of cholesterol, brain SM, and the indicated PS. Brain PS and a mixture that mimics brain PS (16% 18:0, 13% 18:1, 1% 20:4, 3% 22:6 PS) support the binding of α-synuclein. The minimal requirement for membrane association is 18:1 PS in the presence of a polyunsaturated PS (either 20:4 or 22:6). 18:0 PS cannot substitute for 18:1 PS. *B*, binding of α-synuclein to raft-like membranes containing different ratios of 18:1 and 20:4 PS shows that although equimolar concentrations of 18:1 and 20:4 PS (17% each) support membrane association, a larger proportion of 18:1 than 20:4 PS confers more binding than a larger proportion of 20:4 PS. *Numbers* on the *left* indicate size markers (kDa).

The altered gel mobility also persists when using artificial membranes that mimic lipid rafts, confirming that no additional protein is required for the modification or for binding. Interestingly, one previous report has shown a similar shift in the electrophoretic mobility of α-synuclein when using native brain rather than synthetic phospholipid (40). Mass spectrometry analysis of gel-shifted α-synuclein now reveals only unmodified peptides covering >90% of the α-synuclein sequence, which are identical to peptides derived from the unshifted protein. Taken together, these observations strongly suggest that the shift in gel mobility reflects a conformational change rather than a covalent modification. Raft-like membranes containing anionic brain phospholipid thus reproduce features of the interaction with native lipids not previously observed with synthetic liposomes.

What confers the specific binding and modification of α-synuclein by raft-like membranes? Cholesterol does not appear required. In fact, α-synuclein binds more strongly to membranes containing low or no cholesterol than the 33% classically used to produce raft-like membranes. The ability of cholesterol depletion to eliminate the raft association and synaptic localization of α-synuclein thus reflects the dependence of raft integrity on cholesterol, not a direct interaction of cholesterol with α-synuclein. Although it promotes binding to α-synuclein in the absence of cholesterol, sphingolipid also does not appear crucial for the interaction. Similar to previous reports, we find that α-synuclein binding requires acidic phospholipid, in particular PS. However, individual synthetic forms of PS do not support binding. Rather, we find that a very specific combination of 18:1 PS and PS with polyunsaturated acyl chains is required both to bind and shift the electrophoretic mobility of α-synuclein. Supporting the physiological relevance of this combination, ratios reflecting the preponderance of 18:1 over polyunsaturated acyl chains in native membranes are optimal for binding to α-synuclein. Furthermore, the addition of 18:1 PC to 20:4 PS confers binding to

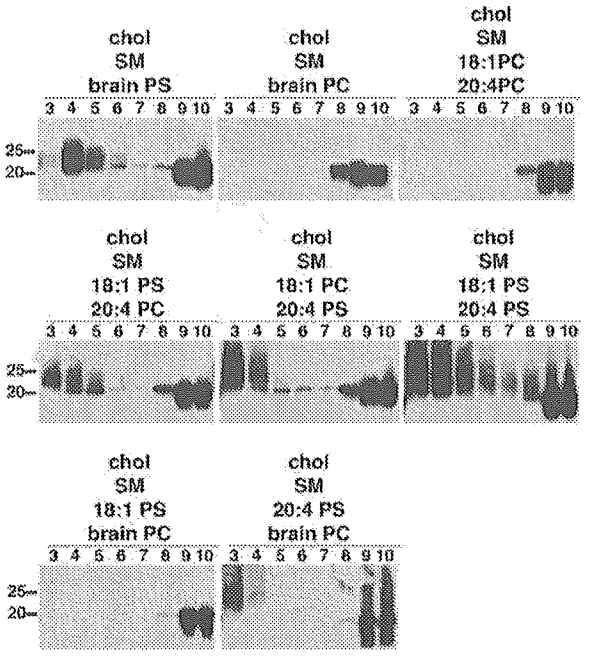

FIG. 8. **Phase transition is required for the binding of α-synuclein to raft-like liposomes.** *Top row*, α-synuclein (100 nM CBP fusion) was incubated with liposomes containing an equimolar mixture of cholesterol (*chol*), brain SM, and the indicated phospholipids, and analyzed as described above. Brain PS but not brain PC or synthetic PC (1:1 ratio of 18:1 and 20:4 PC) support the binding of α-synuclein. *Middle row*, the addition of either 20:4 PC (*left*) or 18:1 PC (*middle*) to 18:1 or 20:4 PS confers binding to α-synuclein. Because the PC head group alone does not support binding, the synthetic forms of PC presumably act by promoting a phase transition between monounsaturated and polyunsaturated fatty acyl chains. *Bottom row*, when added to brain PC, 20:4 PS confers substantially more binding to α-synuclein than 18:1 PS, suggesting specific recognition of the PS head group in the context of polyunsaturated fatty acyl chains. *Numbers* on the *left* indicate size markers (kDa).

α-synuclein even though PC cannot by itself support binding. Conversely, the addition of 20:4 PC to 18:1 PS also promotes binding. The requirement for both mono- and polyunsaturated acyl chains strongly suggests that the interaction of α-synuclein requires membrane with two distinct phases. The requirement for a phase transition may indeed account for the specific association of α-synuclein with rafts, which are considered a liquid-ordered phase distinct from the rest of the liquid-disordered cell membrane (47). Again, previous work has not shown such stringent requirements for the membrane association of α-synuclein, presumably because the specificity observed here depends on the high concentration of nonspecific competitor protein that mimic physiologic conditions in the cytoplasm.

In addition to the requirement for acidic head group and phase transition, we find that α-synuclein binds more strongly to membrane when the polyunsaturated acyl chain of the phospholipid resides on the PS head group. This remarkable specificity presumably reflects an interaction of α-synuclein with both acyl chain and head group. Consistent with the specificity for a particular combination of PS, α-synuclein colocalizes with only a small subset of the PS labeled by annexin V in neurons. Interestingly, the analysis of α-synuclein knock-out mice has shown increases in the level of polyunsaturated fatty acids (49), but the relationship of these changes to the membrane association of α-synuclein remains unclear. In addition, α-synuclein has been reported to bind free oleic acid *in vitro* (50) and to multimerize in the presence of polyunsaturated fatty acids (40), but a specific role for these acyl chains in the membrane association of α-synuclein has not been appreciated before. α-Synuclein has also been reported to bind lipid droplets produced by treatment of cells with oleic acid (18). However, it has remained unclear whether oleic acid simply induces lipid droplets to which α-synuclein can bind. Our results suggest the alternative explanation that α-synuclein interacts directly with the oleoyl side chain of phospholipid in the droplets.

In conclusion, we find that the membrane association of α-synuclein depends on the recognition of phospholipid head group and acyl chain, including phase transition and specific side chain composition. The assay we used to identify these requirements was designed to reproduce the interaction between α-synuclein and native membranes. Thus, the combination of membrane properties identified using this assay presumably contributes to the specific presynaptic localization of α-synuclein. To understand the normal function of this protein as well as its role in PD, future work will need to evaluate the membrane association of α-synuclein in the physiological context of the nerve terminal.

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