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 synaptical 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 acid 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 acid 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 idopathic 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 synapases 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...
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 binding of α-synuclein to synthetic PS with single defined acyl chains do not support trast to previous studies, however, our assay reveals specific.

**sn-glycero-3-phosphoserine (18:0 PS),**

dylethanolamine, 1,2-distearoyl-

respectively. Cholesterol, brain sphingomyelin (SM), brain PS, egg 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-diaryl ether-sn-glycero-3-phosphoserine (18:0 PS), 1,2-dioleoyl-sn-glycero-3-phosphoserine (18:1 PS), 1,2-dilauridion-snglycero-3-phosphoserine (20:4 PS), and 1,2-dioleosahexaenoyl-sn-glycero-3-phosphoserine (22:4 PS) were obtained from Avanti Polar Lipids (Alabaster, AL).

**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-diaryl ether-sn-glycero-3-phosphoserine (18:0 PS), 1,2-dioleoyl-sn-glycero-3-phosphoserine (18:1 PS), 1,2-dilauridion-sn-glycero-3-phosphoserine (20:4 PS), and 1,2-dioleosahexaenoyl-sn-glycero-3-phosphoserine (22:4 PS) were obtained from Avanti Polar Lipids (Alabaster, AL).

**Molecular Biology and Cell Culture—**The construction of a 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% CO₂. 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 Na₃VO₄, 10 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethanesulfonyl 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 × 10⁶) 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.

**Protease 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 mg 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 pellet 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, and electrophoresed 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 35%/30% sucrose interface, solubilized with 1% CHAPS, and the micelles were removed by centrifugation through a 10-kDa molecular mass cutoff Amicon Ultra filter (Millipore, Bedford, MA), and the buffer exchanged to 10 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). Mass spectrometric 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 CaCl₂ during the last 15 min of incubation with secondary antibodies. Fluorescent images were obtained 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.
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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. 2A 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. 2B), 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. 3A). However, a substantial proportion of CD55 remains intact even after prolonged incubation in proteinase K (Fig. 3A), raising questions about the extent of digestion. Resistance to proteolysis could simply reflect the presence of CD55 on the luminal face of isolated DRMs, inaccessible to PK. Luminal 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. 3B). 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. 3A), 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. 3A)
raft-like liposomes (Fig. 4C). 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 living 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. 5A). 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. 5B).

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. 6A). 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. 6B). 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. 6B).

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 these multiple studies dem-
demonstrating 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. 7A, 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. 7A (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. 7A, 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. 7B (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. 7B, 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. 7B). 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. 6A) 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
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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.

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. 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 Ca2+) 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).
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 /H9251/-synuclein when using native brain rather than synthetic phospholipid (40). Mass spectrometry analysis of gel-shifted /H9251/-synuclein now reveals only unmodified peptides covering 90% of the /H9251/-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 /H9251/-synuclein by raft-like membranes? Cholesterol does not appear required. In fact, /H9251/-synuclein binds more strongly to membranes containing low or no cholesterol than the 33% classically used to produce raft-like cholesterol. The ability of cholesterol depletion to eliminate the raft association and synaptic localization of /H9251/-synuclein thus reflects the dependence of raft integrity on cholesterol, not a direct interaction of cholesterol with /H9251/-synuclein. Although it promotes binding to /H9251/-synuclein in the absence of cholesterol, sphingolipid also does not appear crucial for the interaction. Similar to previous reports, we find that /H9251/-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 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).

**FIG. 7. /alpha-Synuclein binding requires PS with both mono- and polyunsaturated fatty acyl chains.** A, /alpha-synuclein (/alpha-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 /alpha-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 /alpha-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).
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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 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.

REFERENCES

1. Polymeropoulos, M. H., Lavedan, C., Leruy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekhararappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Davovius, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) Science 276, 2045–2047

2. Kruger, R., Kuhn, W., Muller, T., Wotailla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J. T., Schols, L., and Ries, O. (1998) Nat. Genet. 18, 106–108

3. Zarranz, J. J., Alegre, J., Gomez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoennicka, J., Rodriguez, O., Attares, B., Llorren, V., Gomez Tortosa, E., del Ser, T., Munoz, D. G., and de Yebenes, J. G. (2004) Ann. Neurol. 55, 164–173

4. Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kuchar, J., Hofn, H. M., Duff, R., Manderson, D., Kuki, E., Arai, H., Simms, K., and Lincoln, S. C., 18:1 PS, suggesting specific recognition of the PS head group in the context of polyunsaturated fatty acid chains. Numbers on the left indicate size markers (kDa).

5. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6469–6473

6. Spillantini M. G., Crowther, R. A., Jakes, R., Cairns, N. J., Lantos, P. L., and Goedert, M. (1998) Neurosci. Lett. 251, 205–208

7. Galvin, J. E., Uryu, K., Lee, V. M., and Trojanowski, J. Q. (1998) Proc. Natl. Acad. Sci. U. S. A. 96, 13450–13455

8. Maroteaux, L., Campaillat, J. T., and Scheller, R. H. (1988) J. Neurosci. 8, 2804–2815

9. Withers, G. S., George, J. M., Banker, G. A., and Clayton, D. F. (1997) Dev. Brain Res. 99, 87–94

10. Murphy, D. D., Ruetter, S. M., Trojanowski, J. Q., and Lee, V. M. (2000) J. Neurosci. 20, 3214–3220

11. Abebe, J., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W. H., Castillo, A., Kretzschmar, H. A., and Haass, C. (2000) J. Biol. Chem. 275, 26292–26294

12. Jensen, P. H., Nielsen, M. S., Jakes, R., Dotti, C. G., and Goedert, M. (1998) J. Biol. Chem. 273, 26292–26294

13. Cole, N. B., Murphy, D. D., Grider, T., Ruetter, S., Brasenlaine, D., and Nussbaum, R. L. (2002) J. Neuror. 22, 8797–8807

14. Udaka, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D. A., Kondo, J., Ibara, Y., and Saito, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11282–11286

15. George, J. M., Jin, H., Woods, W. S., and Clayton, D. F. (1995) Neurobiol. 15, 361–372

16. Iwai, A., Masliah, E., Yoshimoto, M., Ue, N., Flanagan, L., de Silva, H. A., Kittel, A., and Saito, T. (1998) Neurobiol. 14, 467–475

17. Kebbe, P. J., Neumann, M., Oomen, L. M., Muller, V., Schindzielorz, A., Okochi, M., Leimer, U., van Der Putten, H., Probst, A., Kramer, E., Kretzschmar, H. A., and Haass, C. (2000) J. Neurosci. 20, 6365–6373

18. Cole, N. B., Murphy, D. D., Grider, T., Ruetter, S., Brasenlaine, D., and Nussbaum, R. L. (2002) J. Neurosci. 22, 8797–8807

19. Outeiro, T. F., and Lindquist, S. (2003) Science 302, 1772–1775

20. Davidovson, W. S., Jonas, A., Clayton, D. F., and George, J. M. (1998) J. Biol. Chem. 273, 9443–9449

21. Kebbe, P. J., Kehal, E., Russel, R., and Browne, G. (2001) J. Mol. Biol. 310, 4061–4073

22. Jo, E., Fuller, N., Rand, R. P., St. George-Hyslop, P., and Fraser, P. E. (2002) J. Mol. Biol. 315, 799–807

23. Chandra, S., Chen, X., Rizo, J., Jahn, R., and Sudhof, T. C. (2003) J. Biol. Chem. 278, 15313–15318

24. Fortin, D. L., Troyer, M. D., Nakamura, K., Kuhle, S., Anthony, M. D., and Edwards, R. H. (2004) J. Neuror. 24, 6715–6723

25. Edidin, M. (2003) Annu. Rev. Biophys. Biomol. Struct. 32, 257–283

26. Jo, E., McLaurin, J., Yip, C. M., St. George-Hyslop, P., and Fraser, P. E. (2009) J. Biol. Chem. 275, 34328–34334

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 PC 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 acid chains. Bottom row, when added to brain PC, 20:4 PC 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 acid chains. Numbers on the left indicate size markers (kDa).

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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27. Narayanan, V., and Scarlata, S. (2001) *Biochemistry* **40**, 9927–9934
28. Higgins, D., and Banker, G. A. (1998) in *Culturing Nerve Cells* (Banker, G. A., and Goslin, K., eds) 2nd Ed., pp. 37–78, MIT Press, Cambridge, MA
29. Hutner, W. B., Schiebler, W., Greengard, P., and De Camilli, P. (1983) *J. Cell Biol.* **96**, 1374–1388
30. Huttner, W. B., and Jahn, R. (1994) in *Cell Biology, A Laboratory Handbook* (Celis, J. E., ed) Vol. 1, pp. 567–574, Academic Press, San Diego, CA
31. Field, K. A., Holowka, D., and Baird, B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 9201–9205
32. Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000) *J. Biol. Chem.* **275**, 26515–26522
33. Nakamura, T., Yamashita, H., Nagano, Y., Takahashi, T., Avraham, S., Avraham, H., Matsumoto, M., and Nakamura, S. (2002) *FEBS Lett.* **521**, 190–194
34. Ellis, C. E., Schwartzberg, P. L., Grider, T. L., Fink, D. W., and Nussbaum, R. L. (2001) *J. Biol. Chem.* **276**, 3879–3884
35. Veatch, S. L., and Keller, S. L. (2003) *Biophys. J.* **85**, 3074–3083
36. Perrin, R. J., Woods, W. S., Clayton, D. F., and George, J. M. (2001) *J. Biol. Chem.* **276**, 41958–41962
37. Sharon, R., Bar-Joseph, I., Frosch, M. P., Walsh, D. M., Hamilton, J. A., and Selkoe, D. J. (2003) *Neuron* **37**, 583–595
38. Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baha, M., Iwatsubo, T., Meijer, L., Kuhle, P. J., and Haass, C. (2000) *J. Biol. Chem.* **275**, 390–397
39. Shimura, H., Schlossmacher, M. G., Hattori, N., Frosch, M. P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K. S., and Selkoe, D. J. (2001) *Science* **293**, 263–269
40. Nakamura, T., Yamashita, H., Takahashi, T., and Nakamura, S. (2001) *Biochem. Biophys. Res. Commun.* **280**, 1085–1092
41. Ahn, B. H., Ihm, H., Kim, S. Y., Sung, Y. M., Lee, M. Y., Choi, J. Y., Wolzin, B., Chang, J. S., Lee, Y. H., Kwon, T. K., Chung, K. C., Yoon, S. H., Hahn, S. J., Kim, M. S., Jo, Y. H., and Min do, S. (2002) *J. Biol. Chem.* **277**, 12354–12342
42. Fridriksson, E. K., Shipkova, P. A., Sheets, E. D., Holowka, D., Baird, B., and McLafferty, F. W. (1999) *Biochemistry* **38**, 8056–8063
43. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 111–136
44. Frosch, M. P., Hart, L. J., and Selkoe, D. J. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8580–8585
45. Phillips, B., and Solberg, P. L. (2003) *J. Biol. Chem.* **278**, 49874–49881
46. Sharon, R., Bar-Joseph, I., Mirick, G. E., Serhan, C. N., and Selkoe, D. J. (2003) *J. Biol. Chem.* **278**, 49874–49881
47. Sharon, R., Goldberg, M. S., Bar-Joseph, I., Betensky, R. A., Shen, J., and Selkoe, D. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 9110–9115
