Cytosolic Proteins Regulate α-Synuclein Dissociation from Presynaptic Membranes*

Sabine Wislet-Gendebien, Cheryl D'Souza, Toshitaka Kawarai, Peter St George-Hyslop, David Westaway, Paul Fraser, and Anurag Tandon

From the Centre for Research in Neurodegenerative Diseases, University of Toronto, Toronto, Ontario M5S 3H2, Canada

Intracellular accumulation of insoluble α-synuclein in Lewy bodies is a key neuropathological trait of Parkinson disease (PD). Neither the normal function of α-synuclein nor the biochemical mechanisms that cause its deposition are understood, although both are likely influenced by the interaction of α-synuclein with vesicular membranes, either for a physiological role in vesicular trafficking or as a pathological seeding mechanism that exacerbates the propensity of α-synuclein to self-assemble into fibrils. In addition to the α-helical form that is peripherally-attached to vesicles, a substantial portion of α-synuclein is freely diffusible in the cytoplasm. The mechanisms controlling α-synuclein exchange between these compartments are unknown and the possibility that chronic dysregulation of membrane-bound and soluble α-synuclein pools may contribute to Lewy body pathology led us to search for cellular factors that can regulate α-synuclein membrane interactions. Here we reveal that dissociation of membrane-bound α-synuclein is dependent on brain-specific cytosolic proteins and insensitive to calcium or metabolic energy. Two PD-linked mutations (A30P and A53T) significantly increase the cytosol-dependent α-synuclein off-rate but have no effect on cytosol-independent dissociation. These results reveal a novel mechanism by which cytosolic brain proteins modulate α-synuclein interactions with intracellular membranes. Importantly, our finding that α-synuclein dissociation is up-regulated by both familial PD mutations implicates cytosolic cofactors in disease pathogenesis and as molecular targets to influence α-synuclein aggregation.

α-Synuclein (α-syn) is a member of a multigene synuclein family that is highly abundant in presynaptic terminals of mammalian brain (1, 2). The function of α-syn is poorly resolved, although it is attributed with wide ranging roles in vesicular trafficking and vesicle biogenesis and as a molecular chaperone (3). α-Syn is also implicated in a broad spectrum of neurodegenerative disorders collectively named synucleinopathies, being a primary component of Lewy bodies (4), and as a fragment in Alzheimer disease plaques (5). In a small number of pedigrees, autosomal-dominant inheritance of Parkinson disease (PD) is linked to either multiplication of the normal α-syn gene or to one of three missense mutations (A30P, E46K, and A53T) (6, 7).

In vitro studies suggest that α-syn is natively unfolded in aqueous solution, and exposure to lipids stabilizes the amino terminus in an amphipathic α-helix that aligns polar and non-polar residues into opposing orientations (8–11). Presumably, this secondary structure confers the lipid-binding properties for direct membrane interaction such that purified recombinant α-syn can bind to small diameter artificial vesicles rich in acidic phospholipids (8, 12), to purified synaptic vesicles (13, 14), and to membranes within intact cells (15). Most studies examining α-syn membrane binding have used either artificial phospholipids or purified membranes, without consideration to the potential regulatory function of soluble or membrane factors that may be pertinent to disease progression. Previous investigations were equivocal on whether α-syn mutations (A30P and A53T) affect membrane interactions; the A30P or A53T mutation had little or no effect on α-syn binding (16, 17), the A53T mutation reduced (8) or increased (18, 19) the membrane binding, or the A30P had reduced binding to purified synaptic vesicles (14, 20, 21). Further complexity may also arise from reversible membrane interaction, akin to that of cytosolic regulators of vesicular trafficking whose direct or indirect association with vesicle membranes is necessarily transient (e.g. exocytosis: NSF, α/β-SNAP, rab3, rabphilin, synapsin, CAPS; endocytosis: clathrin/adaptor, dynamin) (22–24). Association and dissociation is coupled to the vesicle life cycle, dictated by cation fluxes, nucleotide triphosphate cleavage, and post-translational modifications. In the case of rab3, an additional mechanism governs its membrane attachment. Another cytosolic protein, GDP-dissociation inhibitor, is essential to extract rab3 off exocytosing vesicles and deliver it to newly synthesized vesicles (25–27).

It is unknown whether α-synuclein exchange occurs between soluble and membrane compartments or even whether α-syn can dissociate from reconstituted or biological membranes. The possibility that chronic dysregulation of subcellular α-synuclein pools may contribute to PD and other synucle-
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A. Dissociation assay

Step 1

Step 2

Co-Incubate
Acceptors/Donors

Step 3

Separate soluble and membrane fractions

Centrifuge

Measure α-syn in acceptor fraction

Tg Synaptosomes

α-syn WT, A30P or A53T

Donor (Tg human α-syn)

α-syn deficient mouse brain

α-syn cytosol

Acceptors (α-syn deficient)

FIGURE 1. A, α-syn dissociation assay. Step 1, synaptosomes and cytosol were prepared separately from brains of Tg mice expressing the human α-syn (WT, A30P, or A53T) and α-syn deficient mice, respectively. Step 2, following hypotonic lysis of synaptosomes, the washed membranes (α-syn donor) were co-incubated with α-syn cytosol (α-syn acceptor). Step 3, membrane and cytosolic fractions were separated by centrifugation and the supernatants were analyzed by Western blotting. B, distribution of α-syn in acceptor and donor fractions. Western blots showing total synaptosomal α-syn (detected with anti-α-syn antibody Syn-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and synaptophysin recovered in either supernatant (S) or pellet (P) following resuspension of intact synaptosomes in isotonic buffer, hypotonic buffer, or hypotonically lysed synaptosomes (Donor fraction) washed three times and resuspended in isotonic buffer for 10, 20, or 30 min. As expected, no α-syn was detected in brain cytosol from α-syn deficient mice (acceptor fraction: knockout cytosol).

B. Acceptor and Donor fractions

Hypotonically lysed and washed

KO cytosol

Isotonic buffer

Hypotonic buffer

10 min

20 min

30 min

α-synuclein

GAPDH

Synaptophysin

The human α-syn gene containing a eukaryotic Kozak initiation sequence (GCCGCCACC) (28, 29) upstream of the start codon was ligated into a linearized cos-Tet expression vector containing the Syrian hamster prion protein promoter gene (30, 31). A30P and A53T α-syn mutant transgenes were generated using the Quick change kit (Stratagene). Following microinjection into fertilized oocytes of FVB/N mice, positive founders were identified by amplification of genomic DNA and bred to FVB wild-type mice. To generate α-syn Tg mice lacking endogenous murine α-syn, the α-syn Tg mice were crossed with SNCA+/− mice (Jackson Laboratories). F1 offspring positive for α-syn transgene were backcrossed and F2 progeny were selected for the presence of the neomycin resistance gene and the absence of the endogenous α-syn gene as described previously (32). All animal experiments were performed according to guidelines established in the Canadian Guide for the Care and Use of Laboratory Animals.

Synaptosome Preparation—Synaptosomes were prepared as described (26, 33). Briefly, brains from mice (A30P, A53T, and Wt transgenic mice) were dissected and homogenized with 10 strokes at 500 rpm, in ice-cold buffer A (320 mM sucrose, 1 mM EGTA, 5 mM HEPES (pH 7.4)) and centrifuged at 3000 × g for 10 min. The supernatant was spun for 10 min at 14,000 × g and the pellet (P2) resuspended in buffer A. The P2 fraction was loaded onto a discontinuous Ficoll gradient (13, 9, and 5% in buffer A) and centrifuged for 35 min at 35,000 × g. The 13–9% interface, containing intact synaptosomes, was resuspended in buffer B (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 mM NaHCO3, 1.2 mM Na2HPO4, 1 mM MgCl2, 1 mM EGTA, and 10 mM glucose) and spun at 14,000 × g for 10 min. The pellet was hypotonically lysed by two washes in buffer C (10 mM HEPES, 18 mM KOAc, pH 7.2), spun at 14,000 × g for 10 min, and resuspended in buffer D (25 mM HEPES, 125 mM KOAc, and 2.5 mM MgCl2). After centrifugation (14,000 × g for 10 min), synaptosomes were resuspended in buffer D and incubated (as indicated in each figure legend) with or without α-syn deficient brain or liver cytosol before separating membrane and supernatant by centrifugation at 14,000 × g for 10 min. The supernatant was centrifuged at 100,000 × g to remove trace contaminating elements such as broken membranes or dissociated synaptic vesicles before quantifying soluble α-syn by Western blotting.

Cytosol Preparation from α-Syn Knock-out and Non-transgenic Mice—Mouse brains were homogenized in 10 volumes of homogenizing buffer (1 mM sucrose, 1 mM KOAc, 100 mM MgOAc, 1 mM HEPES (pH 7.4)). The homogenate was spun at
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A. Cytosol dependent α-syn dissociation

| Cytosol (mg/ml) | α-syn | Synaptophysin | Cytosol |
|----------------|-------|---------------|---------|
| 0.015          |       |               |         |
| 0.05           |       |               |         |
| 0.15           |       |               |         |
| 0.5            |       |               |         |
| 1.5            |       |               |         |

B. Cytosol concentration effect

C. Murine α-syn

15,000 × g for 10 min and the supernatant was then centrifuged at 100,000 × g for 1 h. The supernatant was dialyzed 4 times with cold dialysis buffer (1 mM KOAc, 1 M HEPES (pH 7.2)) for 45 min each, centrifuged at 28,000 × g for 25 min, and stored at −80 °C. Protein concentration was determined by BCA protein assay (Pierce).

Western Blotting—Proteins were boiled briefly in loading buffer (10% v/v glycerol; 0.05 M Tris (pH 6.8), 2% SDS, bromphenol blue, and 2.5% v/v β-mercaptoethanol) and separated by electrophoresis using 12% Tris-glycine polyacrylamide gels. Proteins were transferred to nitrocellulose (Life Sciences) and probed by Western blotting using: antibodies against α-syn (monoclonals 211 and Syn-1, Neomarkers), our own rabbit polyclonal (LWS1, 1:1000) raised to a 24-mer α-syn-specific peptide, glycerolphosphate dehydrogenase (monoclonal 6C5, Biodesign), or synaptophysin (monoclonal, Biodesign). Bound horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma) were revealed by chemiluminescence using ECL Plus (GE Healthcare) and quantified with a Storm 860 fluorescent imager and ImageQuant software (GE Healthcare). Statistical comparisons were done with GraphPad InStat software using Student’s t test for comparisons between two groups or ANOVA (Bonferroni’s test) for multiple comparisons.

RESULTS

Cytosolic Proteins Increase α-Syn Dissociation from Membrane—To identify regulators of α-syn solubility, we assessed the effects of cytosolic proteins on the interaction of α-syn with membranes. We measured α-syn dissociation from synaptosomal membranes prepared from brains of transgenic mice expressing human α-syn as the donor of membrane-bound α-syn in the absence or presence of brain cytosol derived from α-syn-deficient mice as the acceptor soluble fraction (Fig. 1A). Intact synaptosomes from α-syn transgenic mouse brain suspended in hypotonic buffer release ~30% of their total α-syn content, which comprises the soluble cytoplasmic pool (Fig. 1B). The remaining α-syn is stably associated with the membrane fraction following resuspension in isotonic buffer and is entirely susceptible to membrane disruption by detergents. The release of membrane-derived α-syn into the supernatant was markedly increased by the addition of cytosol, whereas integral membrane proteins, such as synaptophysin, were retained by the membrane pellet (Fig. 2A). Preincubation of membranes with antiserum raised to α-syn inhibited α-syn dissociation, in contrast to anti-synaptophysin antibodies or preimmune serum, neither of which had any effect (Fig. 2B). The ability to dissociate from presynaptic membranes was not exclusive to human α-syn, because endogenous α-syn from non-transgenic mice also exhibited similar cytosol-dependent dissociation from presynaptic membranes (Fig. 2C).

Comparable levels of a nonspecific protein, bovine serum albumin (BSA), did not affect α-syn solubility (Fig. 3A). However, the cytosolic activity was heat- and protease-labile as cytosol preincubated at 95 °C or pre-digested with trypsin failed to induce α-syn dissociation, suggesting that this process...
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The activities of brain and liver cytosol were compared. Liver-derived cytosol showed some weak activity only at the highest concentration tested, whereas cytosolic brain proteins regulate α-syn dissociation from membranes. A, cytosolic activity is heat and protease labile. Increasing non-specific protein concentration with bovine serum albumin (BSA) did not induce additional α-syn dissociation compared to the "no cytosol" treatment (0 mg/ml cytosol) (one-way ANOVA, p > 0.9, n = 4). Pre-exposure of brain cytosol to either heating for 3 min at 95 °C (heat-inactivated cytosol, HIC) or to trypsin (Trp) digestion completely eliminated the ability of 1.5 mg/ml cytosol (C) to induce α-syn dissociation (one-way ANOVA, p > 0.8, n = 4 – 8). For the trypsin experiments, proteolytic activity was terminated with trypsin inhibitor prior to the incubation with membranes, as shown by the partial rescue after half-dilution with untreated brain cytosol (Trp + C). ***, p < 0.01 relative to no cytosol condition (one way ANOVA, n = 4; Bonferroni's multiple comparison test). B, α-syn dissociation activity is concentrated in brain cytosol. The activities of brain and liver cytosol were compared. Liver-derived cytosol showed some weak activity only at the highest concentration tested, whereas brain cytosol induced significant α-syn dissociation at 6-fold lower concentration 0.5 mg/ml (***, p < 0.0001, one-way ANOVA, n = 4; Bonferroni’s multiple comparison test compared with 0 mg/ml cytosol condition). C, cytosol-dependent α-syn dissociation is influenced by temperature. Top panel, Western blot showing α-syn dissociation in presence or absence of cytosol during 10-min incubation at various temperatures (4, 16, 25, and 37 °C). Bottom panel, mean α-syn intensity and S.E. from five independent experiments and normalized to α-syn dissociation with 1.5 mg/ml cytosol (one-way ANOVA, p < 0.0001, n = 5; Bonferroni’s multiple comparison test revealed that temperature significantly increased cytosol dependent α-syn dissociation). No significant correlation was observed with temperature change in absence of cytosol (one way ANOVA, p > 0.5, n = 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 3. Cytosolic brain proteins regulate α-syn dissociation from membranes. A, cytosolic activity is heat and protease labile. Increasing non-specific protein concentration with bovine serum albumin (BSA) did not induce additional α-syn dissociation compared the "no cytosol" treatment (0 mg/ml cytosol) (one-way ANOVA, p > 0.9, n = 4). Pre-exposure of brain cytosol to either heating for 3 min at 95 °C (heat-inactivated cytosol, HIC) or to trypsin (Trp) digestion completely eliminated the ability of 1.5 mg/ml cytosol (C) to induce α-syn dissociation (one-way ANOVA, p > 0.8, n = 4 – 8). For the trypsin experiments, proteolytic activity was terminated with trypsin inhibitor prior to the incubation with membranes, as shown by the partial rescue after half-dilution with untreated brain cytosol (Trp + C). ***, p < 0.01 relative to no cytosol condition (one way ANOVA, n = 4; Bonferroni’s multiple comparison test). B, α-syn dissociation activity is concentrated in brain cytosol. The activities of brain and liver cytosol were compared. Liver-derived cytosol showed some weak activity only at the highest concentration tested, whereas brain cytosol induced significant α-syn dissociation at 6-fold lower concentration 0.5 mg/ml (***, p < 0.0001, one-way ANOVA, n = 4; Bonferroni’s multiple comparison test compared with 0 mg/ml cytosol condition). C, cytosol-dependent α-syn dissociation is influenced by temperature. Top panel, Western blot showing α-syn dissociation in presence or absence of cytosol during 10-min incubation at various temperatures (4, 16, 25, and 37 °C). Bottom panel, mean α-syn intensity and S.E. from five independent experiments and normalized to α-syn dissociation with 1.5 mg/ml cytosol (one-way ANOVA, p < 0.0001, n = 5; Bonferroni’s multiple comparison test revealed that temperature significantly increased cytosol dependent α-syn dissociation). No significant correlation was observed with temperature change in absence of cytosol (one way ANOVA, p > 0.5, n = 5). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
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α-Syn Dissociation Is Not Regulated by Ca<sup>2+</sup> or by Nucleotide Triphosphates—Because many nerve terminal functions are subject to regulation by Ca<sup>2+</sup> influx and the availability of metabolic energy, we examined whether α-syn translocation is affected by changes in these parameters. No significant differences on α-syn dissociation were observed in the presence or absence (with EGTA) of 1 mM Ca<sup>2+</sup> and in the presence of increasing concentrations of cytosol, suggesting a Ca<sup>2+</sup>-independent mechanism (Fig. 5A). Next, we assessed the effects of ATP and GTP, their non-hydrolyzable analogs, and their various metabolic products in our assay. We did not observe any significant changes to α-syn dissociation in the presence of any of these compounds (Fig. 5B), suggesting that α-syn dissociation occurs independently of high energy phosphates.

**DISCUSSION**

Current models of α-syn lipid interaction predict that the amino-terminal portion of α-syn assembles into an amphipathic α-helix that embeds partially into membrane bilayers (8, 10, 11, 16, 34). In accord with this concept, our results show that the majority of synaptosomal α-syn is associated with membranes in vivo, although substantial portion is freely diffusible within the cytoplasm. How these bound and diffusible pools are maintained and whether regulated exchange occurs between these α-syn compartments is unclear. Here, we present evidence that α-syn stably associated with the membrane fraction can be recruited into the soluble fraction in the presence of brain cytosol. This was not due to a detergent action of cytosol because integral membrane proteins, e.g. synaptophysin, remained with the membrane pellet and the process was blocked specifically by antibodies to α-syn. Because the antisera alone had no affect on α-syn distribution in the absence of cytosol, the antibodies likely prevent access of cytosolic factors to membrane α-syn. Moreover, predigestion of brain cytosol with trypsin or preheating at 95 °C eliminated the activity, directly implicating a
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The proteins triggering α-syn dissociation are in limiting quantity in cytosol and are not regenerated under the conditions of our assay. A single exposure to synaptosome membranes was sufficient to deplete cytosolic capacity to extract membrane α-syn so that subsequent incubations with fresh membranes yielded no additional soluble α-syn. In contrast, presynaptic membranes retained ample extractable α-syn, which could be dissociated with subsequent applications of cytosol. While this suggests that the rate and extent of α-syn dissociation is primarily dependent on the availability of cytosolic factors, additional studies will be needed to determine whether some of the α-syn that remains membrane-bound is biochemically distinct from the cytosol-extracted α-syn.

The cytosolic proteins in our experiments are substantially less concentrated than presynaptic cytosol in vivo (estimated at 200–300 mg/ml) as a result of dilution with extraction buffers during isolation and contamination from non-neuronal cells in brain. Arguably, the portion of α-syn that can potentially be mobilized from membranes is likely to be correspondingly greater in intact cells than the 5% shifts we detected. This is compatible with a previous report by Fortin et al. (35) showing that GFP-tagged α-syn expressed in primary hippocampal neurons accumulates at synaptic boutons and that the fluorescence at photobleached boutons recovers rapidly as if α-syn has substantial steady state mobility and can be replenished from adjacent boutons. Neuronal stimulation in the presence of extracellular Ca2+ dispersed the α-syn fluorescence from presynapses in a tetanus toxin-sensitive manner, suggesting that α-syn translocation occurred after exocytosis and that Ca2+ entry per se is an insufficient trigger. We also did not observe any Ca2+ dependence or a direct requirement for nucleotide triphosphate cleavage in inducing α-syn dissociation. Although it is likely that post-exocytotic regulatory aspects are not recapitulated in our assay, our observations demonstrating cytosol-mediated α-syn dissociation may underlie the movement of GFP-α-syn during resting conditions or associated with exocytosis (35). Our results suggest a dynamic model of α-syn interaction

role for specific cytosolic proteins in controlling α-syn solubility. The permissive factors appear to be enriched in brain since 6-fold greater level of liver cytosol was required to achieve equivalent α-syn dissociation as brain cytosol.

4 W. Balch, personal communication.
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A. Cytosolic and total α-syn levels

![Western blot image](image)

FIGURE 7. A. proportion of cytoplasmic and total synuclein is not affected by PD mutations. Top panel, α-Syn expression levels and distribution in cytoplasm were compared between synaptosomes derived from Wt (lanes 1–3), A30P (lanes 4–6), and A53T (lanes 7–9) α-syn Tg mice. A representative Western blot showing α-syn, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and synaptophysin in the supernatant following resuspension of 100 mg (wet tissue weight) intact synaptosomes into isotonic buffer (lanes 1, 4, and 7), hypotonic buffer (lanes 2, 5, and 8), or 1% Triton X-100 (lanes 3, 6, and 9). Bottom panel, quantification of five independent experiments measuring the cytosolic (C; from lanes 2, 5, and 8) and total (T; from lanes 3, 6, and 9) α-syn. No significant differences were observed between Wt and mutant α-syn expression (one-way ANOVA, cytosolic fraction, p = 0.51, n = 5; membrane fraction, p = 0.26, n = 5). B. left panel: α-syn immunoreactivity was compared between pellet fractions of the hypotonically-lysed synaptosomes from A (lanes 2, 5, and 8). Right panel, no significant differences were observed in the amount of membrane bound α-syn in hypotonically-lysed synaptosomes from α-syn Wt and mutant Tg mice (one-way ANOVA, p > 0.8).

with membranes, in which cytosolic proteins control the exchange of membrane-stabilized α-syn with a diffusible form, and may explain some discrepancies in previous studies regarding the proportions of soluble and bound α-syn, which ranged from equal distribution (36, 37) to virtually all soluble (13). The fact that most of the α-syn in synaptosomes is membrane-bound during steady state conditions suggests that net cytosol-induced dissociation is impeded until activation by physiological stimuli and that the equilibrium between membrane and soluble pools is balanced by regulated reassociation to membranes.

The cytosolic activity that mediates α-syn dissociation clearly distinguishes between the Wt α-syn and the PD-associated mutants. Both A30P and A53T mutations doubled the cytosol-induced α-syn off-rate but had no effect on the cytosol-independent dissociation. This was unexpected because several presynaptic proteins are also recruited into the cytosolic buffer along with α-syn, this is a likely scenario since several presynaptic proteins are transiently associated with the membrane and each has a distinct mechanism that regulates its direct or indirect membrane interaction (22–24). These are mediated by either a membrane protein complex (e.g. α/β-SNAP), phosphorylation (e.g. synapsin), Ca2+ (e.g. CAPS), and nucleotide triphosphate hydrolysis (e.g. NSF, dynamin). In particular, there are similarities between the dissociation of α-syn and the behavior of rab3, a GTP-binding protein that undergoes regulated dissociation and association from vesicles with the aid of GDP-dissociation inhibitor (25–27). Rab3 lacks a transmembrane domain and its membrane-attachment is afforded by hydrophobic prenyl groups at its carboxyl terminus that insert into lipid bilayers (41). GDP-dissociation inhibitor likely shelters the prenyl moieties from aqueous cytoplasm dur-
ing extraction of rab3a from membranes. In the case of α-syn, its amino-terminal is predicted to organize into an amphipathic α-helix that permits partial insertion of hydrophobic residues into the lipid bilayer (11). Regulated dissociation from membranes would require either unfolding of the α-helix or segregation of those hydrophobic residues. Both mechanisms are compatible with a requirement for a cognate cytosolic factor that regulates α-syn solubility. Notably, the differential effect imparted by both PD-linked α-syn mutations relative to Wt α-syn in the presence of cytosol implicates the as yet undefined cytosolic activity in the pathogenic process. Our approach described here to assess α-syn membrane interactions can be used to identify and characterize the cytosolic activity that modulates α-syn sequestration into and exchange between subcellular compartments. Importantly, the underlying mechanism may be amenable to experimental manipulation as a means of influencing α-syn solubility in vivo so as to prevent its pathogenic accumulation.

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