Constitutive Phosphorylation of the Parkinson’s Disease Associated α-Synuclein*

(Received for publication, August 30, 1999, and in revised form, October 1, 1999)

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α-Synuclein has been implicated in the pathogenesis of Parkinson’s disease, since rare autosomal dominant mutations are associated with early onset of the disease and α-synuclein was found to be a major constituent of Lewy bodies. We have analyzed α-synuclein expression in transfected cell lines. In pulse-chase experiments α-synuclein appeared to be stable over long periods (t½ 54 h) and no endoproteolytic processing was observed. α-Synuclein was constitutively phosphorylated in human kidney 293 cells as well as in rat pheochromocytoma PC12 cells. In both cell lines phosphorylation was highly sensitive to phosphatases, since okadaic acid markedly stabilized phosphate incorporation. Phosphoamino acid analysis revealed that phosphorylation occurred predominantly on serine. Using site-directed mutagenesis we have identified a major phosphorylation site at serine 129 within the C-terminal domain of α-synuclein. An additional site, which was phosphorylated less efficiently, was mapped to serine 87. The major phosphorylation site was located within a consensus recognition sequence of casein kinase 1 (CK-1). In vitro experiments and two-dimensional phosphopeptide mapping provided further evidence that serine 129 was phosphorylated by CK-1 and CK-2. Moreover, phosphorylation of serine 129 was reduced in vivo upon inhibition of CK-1 or CK-2. These data demonstrate that α-synuclein is constitutively phosphorylated within its C terminus and may indicate that the function of α-synuclein is regulated by phosphorylation/dephosphorylation.

Beside Alzheimer’s disease, Parkinson’s disease (PD)1 is the most common neurodegenerative disorder (1, 2). Like many other neurodegenerative disorders PD predominantly occurs as a sporadic disease with an onset late during adulthood. However, some rare cases of genetically inherited forms of PD with decreased age of onset have been reported. Two autosomal dominant missense mutations (A53T, A30P) were found to be associated with the α-synuclein gene (3, 4). Mutations in the α-synuclein gene are very rare and certainly not responsible for the majority of familial PD cases (1, 5). Nevertheless, similar to the extremely rare autosomal dominant mutations found within the gene encoding the β-amyloid precursor protein (βAPP) (6), the identification of α-synuclein as one genetic risk factor in familial PD will allow to obtain further and more detailed knowledge on the molecular mechanisms involved in PD. Indeed, shortly after the pathogenic mutations in the α-synuclein gene were identified, α-synuclein was detected within Lewy bodies, the major pathological hallmark of PD (7–10). Moreover, α-synuclein is not only a major component of Lewy bodies in brains of sporadic PD patients, but is also observed in a distinct type of pathological structures in related disorders such as multiple system atrophy (11–16) and Hallervorden-Spatz syndrome (17). Lewy bodies composed of α-synuclein are also observed in some cases of familial Alzheimer’s disease (18). Moreover, the central domain of α-synuclein (non-Aβ component of AD plaques, NAC) has also been observed in senile plaques of Alzheimer’s disease patients (19). These data therefore suggest that the deposition of α-synuclein in Lewy bodies contributes to the progression of PD and other related neurodegenerative disorders.

A common hallmark of many neurodegenerative diseases is the aggregation and filament formation of disease type-specific amyloid molecules (20). Based on our current understanding the aggregation of such amyloidogenic molecules is accelerated by mutations responsible for the familial forms of the respective disease (21). Indeed, α-synuclein forms fibrils in vitro (8, 22–26) and recent evidence indicated that mutations associated with familial PD accelerate fibril formation (8, 22, 23, 26).

Synucleins belong to a gene family composed of the closely related α-, β-, and γ-synucleins (2). Synucleins are small proteins of 120 to 140 amino acids. In the brain only α- and β-synucleins are expressed (14). α- And β-synucleins are enriched at presynaptic terminals in songbirds, rat, and human (27–33). In vitro, α-synuclein binds to brain vesicles (34) and small synthetic unilamellar liposomes (35). Interestingly, PD-associated mutations appear to affect membrane association of α-synuclein (34). This may indicate that the lack of vesicle association of α-synuclein could be directly associated with aggregation and filament formation of unbound excess amounts of α-synuclein (34). The biological function of synucleins in mammalians is unknown. In songbirds α-synuclein may play a role in synaptic plasticity during song learning (28).

The bovine brain-specific 14-kDa protein, which belongs to the β-synucleins, occurs as a phosphoprotein (36). However, the respective phosphorylation sites and the protein kinases involved have not been identified. Moreover, very little is known about phosphorylation of the PD-associated α-synuclein.
α-Synuclein appears to contain several consensus sequences for protein kinase C (PKC)-mediated phosphorylation (19). Since phosphorylation plays a pivotal role for the functional properties of numerous proteins, we investigated phosphorylation of α-synuclein in transfected cells. We mapped a constitutive phosphorylation site to serine 129 and provide evidence that casein kinase 1 (CK-1) and/or CK-2 but not PKC or PKA may be the responsible kinases phosphorylating α-synuclein in human kidney 293 cells and in rat pheochromocytoma PC12 cells.

MATERIALS AND METHODS

Cell Culture, Transfection, and Generation of Stable Cell Lines—K293 cells were maintained in Dulbecco’s modified Eagle's medium containing Glutamax and 10% fetal calf serum (Life Technologies, Inc.) with penicillin and streptomycin. PC12 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 5% horse serum (Life Technologies, Inc.). K293 cells were transfected using N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roche) and PC12 cells were transfected with FuGene (Roche) according to the supplier’s instructions. K293 and PC12 cells stably expressing α-synuclein cDNAs (cloned into the pcDNA3 vector (Invitrogen)) were selected and maintained in the above mentioned medium containing 500 μg/ml G418 (Life Technologies, Inc.).

Construction of cDNAs—Epitope tagging of the α-synuclein cDNAs with a sequence encoding the FLAG epitope at the N terminus, and introduction of the respective point mutations was carried out by polymerase chain reaction using the full-length α-synuclein cDNA (gift from Dr. J. Hardy) as a template. The cDNAs encoding the FLAG-tagged α-synuclein serine to alanine mutants S9A, S42A, S77A, and S129A were constructed by mutating the respective codons in a two-step polymerase chain reaction procedure.

The cDNA encoding the FLAG-tagged α-synuclein serine to alanine double mutants (S9A/S129A, S42A/S129A, S77A/S129A) were constructed by the same two-step polymerase chain reaction procedure with the α-synuclein S129A cDNA as template. All cDNAs were sequenced to ensure the correct amino acid substitution.

Construction of cDNAs Encoding MBP- and Glutathione S-Transferase-α-Synuclein Fusion Proteins—The full-length cDNA sequence of α-synuclein was amplified by polymerase chain reaction and the resulting DNA fragments were cloned into the XmnI/BamHI restriction sites of pMAL-c2 (New England Biolabs) or into the BamHI/SalI sites of pGEX-5X-1 (Amersham Pharma Biotech), respectively.

Purification of Fusion Proteins and Generation of Polyonal Antibodies—Fusion proteins of the respective α-synuclein domains (amino acids 1–140 or 96–140) were expressed in Escherichia coli DH5α. MBP-α-synuclein fusion protein was purified on amylose resin (New England Biolabs) and glutathione S-transferase-α-synuclein fusion protein was purified on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the supplier’s instruction. MBP-α-synuclein fusion protein was injected into rabbits to raise antisera PEB1. The glutathione S-transferase-α-synuclein fusion protein was injected into rats to raise antisera Syn-C. The monoclonal antibody M2 recognizing the FLAG epitope (DYKDDDDK) was obtained from Sigma. The monoclonal anti-α-synuclein antibody LB509 (10) and antisera APP C4 (gift from Dr. Y. Ihara) directed to the cytoplasmic tail of APP were used to detect α-synuclein and APP, respectively (37).

Metabolic Labeling, in Vivo Phosphorylation, and Immunoprecipitation/Immunoblotting—Stably transfected K293 or PC12 cell lines were grown to subconfluence in 6-cm dishes. After starvation for 2 h at 37 °C, the conditioned medium was then aspirated, cells were washed twice with ice-cold phosphate-buffered saline, and immediately lysed on ice with 10 concentrated RIPA buffer containing 1% SDS, 5% deoxycholic acid, and 10% Triton X-100 for 5 min. After addition of ice-cold phosphate-buffered saline with protease inhibitors (Sigma), cell lysates were centrifuged 10 min at 14,000 ,g, and supernatants were immunoprecipitated with the indicated antibodies for 2 h. Immunoprecipitates were resuspended in sample buffer for 5 min at 65 °C, separated on SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Immobilon). Radiolabeled peptides were visualized by autoradiography. Quantiﬁcation was performed by PhorImaging (Molecular Dynamics) using the ImageQuant program.

Stimulation and Inhibition of Kinases and Phosphatases—To activate PKC and PTK during in vivo labeling with [32P]orthophosphate, phosphorylation was carried out in the presence of 1 μM phorbol 12,13-dibutyrate (Biomol Research Labs Inc., Plymouth Meeting, PA) or 5 μM forskolin (Biomol). To inhibit phosphatase activities, 1 μM okadaic acid (LC Laboratories, Woburn, MA) was used during in vivo labeling (40). To inhibit CK-1 and CK-2, 5 μM newly developed CK-1 inhibitor HD-01, or 50 μM of the CK-2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (Biomol) was added to the incubation media (42).

In Vitro Phosphorylation Assays—Recombinant rat CK-1 (New England Biolabs) and CamKII (New England Biolabs) were used for in vitro phosphorylation assays according to the supplier’s instructions. Recombinant subunits of human CK-2 (gift from Dr. W. Pyerin), and the catalytic subunit of PKA purified from bovine heart (gift from Dr. V. Kinzel) were used in a buffer containing 20 mM Tris, pH 7.5, 5 mM magnesium acetate, 5 mM dithiothreitol. PKC purified from rat brain (Biomol) was assayed in a similar buffer supplemented with 1 μM phorbol 12,13-dibutyrate, 0.5 mM calcium chloride, and 100 μg/ml phosphatidylerine under mixed micellar conditions (43). The activity of each kinase was monitored using histone or phosvitin as control substrates.

Recombinant α-synuclein expressed in E. coli was cleaved from the MBP fusion protein by incubation with factor Xa according to the supplier’s instruction (New England Biolabs). The serine to alanine mutants were purified by immunoprecipitation. Purified α-synucleins were then used in the above described reaction mixtures and the phosphorylation reactions were started by the addition of γ-[32P]ATP. The reactions were stopped by the addition of SDS sample buffer after 30 min at 30 °C.

Phosphoamino Acid Analysis and Phosphopeptide Mapping—Isolated [32P]-labeled α-synuclein was separated by SDS-polyacrylamide gel electrophoresis, electrotransferred onto polyvinylidene difluoride membranes (Millipore), and subjected to one-dimensional phosphoamino acid analysis (44) or two-dimensional phosphopeptide mapping (45). The radiolabeled proteins were hydrolyzed by incubation in 6 N HCl for 90 min at 110 °C. After centrifugation, the supernatants were dried in a SpeedVac concentrator. The resulting pellets were dissolved in pH 2.5 buffer and spotted onto cellulose TLC plates together with unlabeled phosphoamino acids (Serva, Thermo, and TyrP, 1 μg each). High voltage electrophoresis was carried out for 45 min at 20 mA. Radioactive phosphoamino acids were identified by autoradiography and co-migration with the ninhydrin-stained standards.

For phosphopeptide mapping, [32P]-phosphorylated α-synuclein was excised from polyvinylidene difluoride membranes and digested for 24 h at 37 °C with 0.5 μg/ml trypsin (Roche Molecular Biochemicals, sequencing grade) as described previously (46). After two-dimensional separation on cellulose plates, radiolabeled peptides were visualized by autoradiography.

RESULTS

Expression of α-Synuclein in Human Brain and Tissue Culture Cells—In order to analyze α-synuclein expression, we generated an antibody (αSyn-C) to the C-terminal domain of synuclein. To prove that this antibody does not cross-react with the homologous β-synuclein, we purified α- and β-synuclein from human brain (36, 47, 48). Purified human brain synucleins were then detected by immunoblotting with αSyn-C or β-synuclein antibodies (10). αSyn-C exclusively detected

2 L. Meijer, A.-M. W. H. Thunnissen, A. W. White, M. Garnier, M. Nikolic, L.-H. Tsai, J. Walter, K. E. Cleverley, P. C. Salinas, Y.-Z. Wu, J. Biernat, E.-M. Mandelkow, S.-H. Kim, and G. R. Pettit, Chem. Biol., submitted for publication.
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**Fig. 1. Expression of synucleins in human brain, kidney 293 cells (K293), and PC12 cells.**

**a.** purified human brain synucleins (α-syn and β-syn) were identified by immunoblotting using anti-αSyn-C (left panel) or anti-β-synuclein (right panel). Note, that αSyn-C specifically detected α-synuclein with no cross-reactivity to β-synuclein. **b.** stable expression of α-synuclein in tissue culture cells. The indicated cell lines were transfected with the α-synuclein cDNA with and without a FLAG tag. Cell lysates of the indicated lines were immunoprecipitated and detected by immunoblotting with antibodies αSyn-C. Mock transfections (pcDNA3) of the empty vector served as a negative control. Bands labeled by asterisks (*) correspond to rat IgG light chains.

**c.** α-synuclein is stable during long cold chase periods. K293 cells and PC12 cells stably transfected with FLAG-tagged α-synuclein were pulse labeled with [35S]methionine and chased for the indicated time points. Cell lysates were immunoprecipitated with antibody M2. **d.** de novo protein synthesis with cycloheximide. After the indicated periods in the presence of excess amounts of unlabeled methionine. Cell lysates from untransfected K293 cells as well as cell lines stably expressing α-synuclein (Fig. 1a) were pulse-labeled for 2 h and chased for the indicated time points (data not shown). 

**Human kidney 293 cells (K293) as well as rat pheochromocytoma cells (PC12) were stably transfected with the α-synuclein cDNA with and without a FLAG-tag added to the N terminus.** Cell lysates from untransfected K293 cells as well as cell lines stably expressing α-synuclein were immunoprecipitated either with antibody Syn-C or with an anti-FLAG antibody (αM2) and precipitated α-synucleins were detected with antibody Syn-C. In untransfected K293 cells low amounts of the endogenous 19-kDa α-synuclein were observed with antibody Syn-C (Fig. 1b). The signal was strongly augmented when stably transfected cell lines were investigated (Fig. 1b). Cell lines (K293 and PC12) expressing FLAG-tagged α-synuclein showed significant amounts of α-synuclein. Due to the addition of the FLAG-tag the expected shift in the molecular weight is observed (Fig. 1b). α-Synuclein was also identified with an antibody raised to the recombinant full-length protein (FR1) (data not shown).

Pulse-chase experiments were carried out to determine the half-life of α-synuclein. Cells stably expressing α-synuclein were pulse-labeled for 2 h and chased for the indicated time periods in the presence of excess amounts of unlabeled methionine. α-Synuclein was found to be a relatively long-lived protein (Fig. 1c). To further prove these results we inhibited de novo protein synthesis with cycloheximide. After the indicated time points cells were lysed and α-synuclein expression was analyzed by immunoblotting. As shown in Fig. 1d, α-synuclein was stable over 16 h, whereas the short-lived βAPP expressed in the same cells disappeared within the first 4 h. Therefore α-synuclein is remarkably stable and under the conditions used in these experiments, endoproteolysis or NAC generation was not observed (Fig. 1, c and d; and data not shown).

**α-Synuclein Is Constitutively Phosphorylated on Serine Residues**—We next investigated if α-synucleins are phosphorylated in K293 and PC12 cells. Since several potential recognition sites for PKC/PKA-mediated phosphorylation were proposed to be located within the repeat region of α-synuclein (19), cells were incubated with [32P]orthophosphate in the presence or absence of forskolin and phorbol 12,13-dibutyrate to stimulate PKA or PKC, respectively. Cell lysates were immunoprecipitated with αSyn-C (Fig. 2a, left panel) or αM2 (Fig. 2, a, right panel, and b) and phosphorylated synucleins were visualized by autoradiography. Expression of α-synuclein was analyzed by immunoblotting (lower panel) of the same membrane, which was used for autoradiography. In K293 cells transfected with the native α-synuclein cDNA or with the FLAG-tagged cDNA, stimulation of PKC or PKA did not increase phosphate incorporation as compared with controls (Fig. 2a). Successful activation of PKA and PKC was proven by the increased phosphorylation of the presenilin-1 C-terminal fragment (data not shown; Refs. 49–51). Interestingly, treatment with okadaic acid, which blocks protein dephosphorylation by the inhibition of protein phosphatase 1 and 2A leads to a substantial increase of phosphate incorporation (Fig. 2a). However, even when phosphatases were inhibited, stimulation of PKC or PKA did not result in a further increase of phosphate incorporation (Fig. 2a, right panel). Since we could not exclude the possibility that α-synuclein undergoes cell-specific phosphorylation in neuronal cells, we also analyzed α-synuclein phosphorylation in PC12 cells. Again, stimulation of PKC or PKA did not increase phosphate incorporation (Fig. 2b), however, inhibition of dephosphorylation greatly enhanced phos-
phosphorylation site(s) we performed site-directed mutagenesis to alanine. To avoid signals derived from endogenous sequence (at positions 9, 42, 87, and 129) (19), which we mutated.

Lower panel (Fig. 3, lower panel), constitutive phosphorylation of α-synuclein in PC12 cells. Stimulation of PKC/PKA did not increase phosphorylation into α-synuclein of PC12 cells. α-Synuclein was in vivo phosphorylated and isolated as described in α. Longer exposure revealed phosphorylated α-synuclein in cells not treated with OA (data not shown).

To further characterize phosphorylation of α-synuclein, we performed a phosphoamino acid analysis. Cells were incubated with [32P]orthophosphate in the presence and absence of okadaic acid. α-Synuclein was isolated by immunoprecipitation (Fig. 3, lower panel). After acidic hydrolysis, radiolabeled amino acids were identified by one-dimensional electrophoresis. Phosphoamino acid analysis revealed that α-synuclein was predominantly phosphorylated on serine residues in the presence and absence of phosphatase inhibitors (Fig. 3).

Identification of the Phosphorylation Sites—To identify the phosphorylation site(s) we performed site-directed mutagenesis. Four serine residues are present in the human α-synuclein sequence (at positions 9, 42, 87, and 129) (19), which we mutagenized to alanine. To avoid signals derived from endogenous α-synuclein, all cDNAs were FLAG-tagged. The cDNAs were stably transfected into K293 cells and cell lines expressing the recombinant proteins were isolated. Cell lines expressing wild type or the corresponding serine to alanine mutation were incubated with [32P]orthophosphate in the presence or absence of forskolin, phorbol 12,13-dibutyrate (PDBu), and okadaic acid (OA). Cell lysates were immunoprecipitated with antibody α-Syn-C (left panel) or αM2 (right panel) and phosphorylated α-synuclein was visualized by autoradiography. Expression of α-synuclein was monitored by immunoblotting of the membrane with antibody Syn-C after autoradiography (lower panel).

In vitro phosphorylation experiments, K293 cells were in vivo phosphorylated with [32P]orthophosphate. α-Synuclein was isolated by immunoprecipitation (lower panel) and subjected to one-dimensional phosphoamino acid analysis. Asterisk (*) corresponds to polypeptides, which were not fully hydrolyzed.

The lack of a complete inhibition of phosphate incorporation may be due to an additional phosphorylation site. To prove this directly we generated double serine to alanine mutations (S129A/S9A, S129A/S42A, and S129A/S87A) which were transiently transfected into K293 cells. Phosphate incorporation was analyzed as described above. Again, we found a substantial decrease of phosphate incorporation into α-synuclein in cells expressing S129A. There was no further decrease in phosphate incorporation obtained in cells expressing the S129A mutation together with the S9A or S42A mutation (Fig. 4b). However, cells expressing the double mutation S129A/S87A showed a significant further decrease of phosphate incorporation (Fig. 4b).

The use of two sites, which are phosphorylated with different efficiencies is further supported by two-dimensional phosphopeptide mapping. K293 cells stably transfected with the wild-type α-synuclein cDNA or the S87A and S129A mutation were incubated with [32P]orthophosphate in the presence of okadaic acid. Immunoprecipitated α-synuclein was digested with trypsin. Based on a sequence analysis, peptides would be expected, which contain no more than 1 serine residue (data not shown). After electrophoretic and chromatographic separation the resulting cleavage products were identified by autoradiography. One major spot was detected when wild type α-synuclein was analyzed (Fig. 4c, spot 1). This spot was absent when the S129A mutation but not the S87A mutation was expressed. Furthermore, a weak spot present in wild type transfected cells was reduced upon the expression of the S87A mutation (Fig. 4c, spot 2). From these data we conclude that α-synuclein is predominantly phosphorylated at serine residue 129. However, a second serine at position 87 is also used for phosphorylation to some extent.

Identification of Potential Kinases Involved in Phosphorylation of α-Synuclein—To investigate which kinase is involved in the phosphorylation of α-synuclein, we first performed in vitro assays using the purified kinases CK-1, CK-2, PKC, PKA, and CamKII. CK-1 and CK-2 activity was monitored using histone as control substrates (Fig. 5, lower panel, and data not shown). Consistent with the data derived from the in vivo phosphorylation experiments, α-synuclein was not significantly phosphorylated in vitro by PKC or PKA (Fig. 5a). In contrast, CK-1 and CK-2 efficiently phosphorylated α-synuclein in vitro (Fig. 5b). A substantially lower phosphorylating activity was also found for CamKII (Fig. 5b). CK-1 and CK-2 phosphorylated α-synuclein was analyzed by phos-
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Fig. 4. Identification of in vivo phosphorylation site(s) of α-synuclein. a, the 4 serine encoding codons of α-synuclein were individually mutagenized to alanine and the resulting cDNAs were stably transfected into K293 cells. The cell lines were in vivo phosphorylated with [32P]orthophosphate in the presence or absence of OA. Phosphorylated α-synuclein was identified by immunoprecipitation using antibody αM2. Expression of α-synuclein was monitored by immunoblotting of the membrane with αSyn-C after autoradiography. Mutagenesis of serine 129 significantly reduced 32P incorporation into α-synuclein in the presence and absence of OA. For quantitation the ratio between the 32P-labeled band and the α-synuclein band detected by immunoblotting was determined. 100% = phosphate incorporation of K293 cells expressing FLAG-tag wild type α-synuclein without OA treatment. b, mutagenesis of serine residues 129 and 87 further decreased phosphorylation of α-synuclein, whereas all other double mutations showed no effect. Experiments were carried out as described in a except that K293 cells were transiently transfected. Immunoblotting (lower panel) reveals equal transfection efficiencies. c, phosphopeptide mapping demonstrates that serine 129 is a major phosphorylation site. K293 cells expressing wild type α-synuclein or the indicated serine mutations were in vivo phosphorylated with [32P]orthophosphate and α-synucleins were isolated by immunoprecipitation as described above. In vivo phosphorylated α-synuclein was digested with trypsin and the resulting peptides were separated by two-dimensional chromatography. Note that the S129A mutation eliminated spot "1" in the upper part, whereas the S87A mutation affected the weaker spot "2" in the lower part. Arrowhead, start. An additional weakly phosphorylated peptide was obtained (labeled by *).

α-Synuclein has recently been implicated to be associated with PD, since two independent mutations were found to cosegregate with early onset familial PD (3, 4). Moreover, α-synuclein was found to be a major constituent of Lewy bodies, the pathological hallmark of this devastating disease (7–10). Very little is known about the expression and function of α-synuclein under physiological and pathological conditions. Non-human synucleins have been implicated to be major phosphoproteins (36) and human α-synuclein was proposed to be phosphorylated by PKC/PKA within the characteristic KTKEGV repeats (19). We investigated phosphorylation of α-synuclein in vivo, since phosphorylation may play a role in the functional regulation of α-synuclein (19). We found that human α-synuclein is constitutively phosphorylated predominantly on serine residues and that phosphate incorporation cannot be increased by stimulation of PKC/PKA. Under steady state conditions, most of the α-synuclein molecules appeared to be dephosphorylated, since without the treatment with phosphatase inhibitors during the labeling period significantly less phosphorylated α-synuclein was observed. This indicates a tight regulation of α-synuclein phosphorylation/dephosphoryla-
tion and could suggest that repeated phosphorylation and dephosphorylation within the C-terminal domain of α-synuclein occurs in vivo.

We determined a major phosphorylation site at serine residue 129 and a second phosphorylation site at serine 87. The major phosphorylation site at serine 129 as well as the acidic sequence motif required for substrate recognition by CK-1 or CK-2 is also well conserved between several different mammalian species (Fig. 7). Generally, all mammalian α-synucleins bear a C-terminal serine at a site characteristic for each family member: serine 129 in α-synuclein, serine 118 in β-synuclein, and serine 124 in γ-synuclein (Fig. 7). Although these serine residues appear in topologically different positions, they all are flanked by acidic residues EMPSEE for α-synuclein serine 129, EGESYVED for β-synuclein serine 118, and EEAQSGGD for γ-synuclein serine 124. Serine 129 is located within a cognate recognition sequence for CK-1 with a glutamate residue in the +2 position (52, 53). Because serine 129 is followed by two acidic residues in positions +1 and +2, this site might also be recognized by CK-2 in vivo (52, 53). Therefore, serine 129 may be alternatively phosphorylated by CK-1 or CK-2. Based on the conserved sequence motif around the critical serine residue, phosphorylation of these serine residue might

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be a common phenomenon, although it remains to be shown if β- and/or γ-synuclein are phosphorylated by CK-1 and CK-2 as well. Interestingly, at least one regionally expressed human α-synuclein splice variant (NACP 112) serine 129 is one of the internally deleted C-terminal amino acids (54). Therefore this splice variant might not undergo constitutive phosphorylation.

In contrast to the highly conserved serine residue at position 129, human α-synucleins are exceptional with regard to the serine residue at position 87. All other synucleins described to date (including canary synelfin and torpedo synuclein) share an absolutely conserved asparagine at this position (Fig. 7). Therefore serine 87 phosphorylation appears to be a specific for human α-synuclein.

CK-1 and CK-2 are ubiquitously expressed, constitutively active kinases which are localized in the nucleus and the cytosol (55–57). Association of CK-1 and CK-2 with the plasma membrane and the cytoskeleton has also been reported (56, 58–60). The broad specificity and the various cellular protein substrates of CKs suggest that these kinases are involved in diverse regulatory processes. There is evidence that CK-1 and specifically CK-2, are key players in the regulation of neuronal function and other neuronal proteins, including DARPP-32 (66) and shown to phosphorylate a number of synaptic vesicle proteins, like VAMP, SV-2, and p56. This may be specifically interesting since α-synuclein binds to brain vesicles (34) as well as small synthetic unilamellar liposomes (35) and PD-associated mutations appear to affect membrane binding of α-synuclein (34). The CK-1 activity that is associated with synaptic vesicles shares characteristics of the α isofrom and is strongly inhibited by phosphatidylinositol 4,5-bisphosphate (64). In addition, CK-2 is highly expressed in the hippocampal region and rapidly activated during long-term potentiation (65). CK-2 was also shown to phosphorylate neurmodulin and other neuronal proteins, including DARPP-32 (66) and MAP-1B (67). Together, these data may indicate that CK-1 and CK-2 are involved in the regulation of neuronal function and one may speculate that phosphorylation of α-synuclein could affect its binding to membranes. Moreover, CK-1 phosphorylation was reported to be activated in striatogniral neurons where dopaminergic neurons specifically degenerate during PD (41). Therefore, phosphorylation of serine 129 might also be increased particularly in striatogniral neurons, which could play a role for dopamine release and uptake in synaptic vesicles.

Acknowledgments—We thank Drs. J. Hardy and K. Duff for the gift of the α-synuclein cDNA, Drs. K. Ikeda and T. Arai for α-synuclein cDNA, Drs. K. Ikeda and T. Arai for the gift of the α-synuclein cDNA, Drs. K. Ikeda and T. Arai for the gift of the α-synuclein cDNA. We thank Drs. J. Hardy and K. Duff for the gift of the α-synuclein cDNA, Drs. K. Ikeda and T. Arai for the gift of the α-synuclein cDNA, Drs. K. Ikeda and T. Arai for the gift of the α-synuclein cDNA.
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