α-Synuclein Is Phosphorylated by Members of the Src Family of Protein-tyrosine Kinases*

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α-Synuclein (α-Syn) is implicated in the pathogenesis of Parkinson’s Disease, genetically through missense mutations linked to early onset disease and pathologically through its presence in Lewy bodies. α-Syn is phosphorylated on serine residues; however, tyrosine phosphorylation of α-Syn has not been established (1, 2). A comparison of the protein sequence between Synuclein family members revealed that all four tyrosine residues of α-Syn are conserved in all orthologs and β-Syn paralogs described to date, suggesting that these residues may be of functional importance (3). For this reason, experiments were performed to determine whether α-Syn could be phosphorylated on tyrosine residue(s) in human cells. Indeed, α-Syn is phosphorylated within 2 min of pervanadate treatment in α-Syn-transfected cells. Tyrosine phosphorylation occurs primarily on tyrosine 125 and was inhibited by PP2, a selective inhibitor of Src protein-tyrosine kinase (PTK) family members at concentrations consistent with inhibition of Src function (4). Finally, we demonstrate that α-Syn can be phosphorylated directly both in cotransfection experiments using c-Src and Fyn expression vectors and in in vitro kinase assays with purified kinases. These data suggest that α-Syn can be a target for phosphorylation by the Src family of PTKs.

An ~35-amino acid fragment of α-Syn1 was identified initially as a component of amyloid plaques isolated from Alzheimer’s disease brains. The α-Syn gene was cloned and found to code for a protein containing 140 amino acids (5). Subsequently, mutations in α-Syn have been identified in families with autosomal dominant Parkinson’s Disease (6, 7). Additionally, in patients with sporadic PD, α-Syn immunoreactivity is detected in Lewy bodies, the pathologic hallmark of PD (8). Although, α-Syn is linked to the two most common neurodegenerative disorders, its role in the pathogenesis of these diseases is unknown. The recent observation that both mice and flies expressing a human α-Syn transgene recapitulate some characteristics of PD suggests that α-Syn could be involved directly in the development of this disease (9, 10).

Among the three members of the Syn gene family (α, β, γ) α-Syn is the best characterized. It is most highly expressed in presynaptic neurons of the brain with greater abundance in areas of the hippocampus and cortex (3, 11, 12). α-Syn is a small acidic protein containing three discernable regions: an amino-terminal amphipathic repeat region, which can form α-helices; a hydrophobic center region found in amyloid plaques; and an acidic carboxyl-terminal region. In solution, α-Syn takes on a natively unfolded conformation; however, in the presence of small vesicles composed of acidic phospholipids it forms an α-helical structure. This structure is consistent with its observed binding to synaptic vesicles in vivo (13, 14).

Protein-tyrosine phosphorylation is thought to be important in regulating synaptic function and plasticity (15, 16). Although α-Syn was shown recently to be phosphorylated on serine, it has not been determined whether α-Syn is also phosphorylated on tyrosine residues (1, 2). The possibility that α-Syn might be phosphorylated on tyrosine(s) was initially hypothesized primarily because of the conservation of the four tyrosine residues among α-Syn orthologs (3). In addition to the conserved nature of the tyrosine residues in α-Syn, at least one of these residues contains flanking sequences that share homology with established tyrosine phosphorylation sites. Given the importance of tyrosine phosphorylation in the regulation of many cellular processes, we examined whether α-Syn is phosphorylated on tyrosine.

MATERIALS AND METHODS

Cell Culture and Transfections—Human embryonic kidney cells (HEK293T, gift of Dr. David Baltimore, California Institute of Technology, Pasadena, CA) were cultured in DMEM containing 10% fetal bovine serum, 2 mM l-glutamine, 5 mM HEPES, 10 units/ml penicillin, and 10 μg/ml streptomycin. Cells were transfected using CaPO4 (Stratagene) with α-Syn, FynT, Rlk-GFP, and wild-type and mutant c-Src (R295M) expression vectors (17, 18). HEK293 cells (ATCC CRL-1573) stably transfected with human α-Syn were obtained from Dr. Virginia Lee (University of Pennsylvania, Philadelphia, PA) and cultured as above. PP2 was obtained from Calbiochem.

Construction of α-Syn Expression Vectors—α-Syn was isolated by reverse transcriptase-PCR using human lymphoblastoid cell DNA.2 PCR mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). Amino-terminal FLAG-tagged α-Syn was constructed by PCR using the primer sequences GCTCTAGAGCGACCATGTTAACAGGATGACGATAGATATCTA (5’) and CCCTCGAGGCTCTAGATTTGCTAGTTGATA (3’) (Life Technologies, Inc.) by standard methods, and was ligated into pcDNA 3.1 vector (Invitrogen). All constructs were sequenced on both strands by Se- qwright (Houston, TX).

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1 The abbreviations used are: α-Syn, α-synuclein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; PTP, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase; PD, Parkinson’s disease; GFP, green fluorescent protein; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HEK, human embryonic kidney cells.

2 R. L. Nussbaum, unpublished data.
**α-Syn Is Phosphorylated on Tyrosine**

**Purification of α-Syn from Baculovirus—**Human α-Syn was subcloned into pBlueBacHis vector, and virus production and expression in SF9 cells were performed as described (Invitrogen). The cell pellet was resuspended in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM imidazole, and 1X protease mixture (Sigma), and the lysate was boiled for 10 min. The cleared lysate was bound to Talon resin (CLONTECH), and α-Syn was eluted with 1M imidazole. α-Syn-containing fractions were identified by SDS-PAGE, pooled, dialyzed against 20 mM Tris-HCl, pH 7.5 and 100 mM NaCl and stored at −80°C.

**Western Analysis and Immunoprecipitations—**Transiently transfected HEK293T cells and stably transfected HEK293T cells were harvested in CHAPS lysis buffer (0.5% CHAPS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 2 mM EGTA, 0.5% sodium orthovanadate, and 1X Protease inhibition mixture) or RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EGTA, and 0.5% sodium orthovanadate), snap frozen in a dry ice/ethanol bath and stored at −80°C. Protein was quantified using the BCA protein assay kit (Pierce) using bovine serum albumin as a standard.

FLAG-α-Syn was isolated from cell lysates by immunoprecipitation using an anti-FLAG M2 affinity resin (Sigma), whereas untagged α-Syn was isolated using a monoclonal antibody (202) cross-linked to protein A-agarose beads (Roche Molecular Biochemicals) using dimethylpime-}

**Metabolic Labeling and Phosphoamino Acid Analysis—**HEK293T cells transiently transfected with FLAG-α-Syn were labeled with H$_2$-[32P]PO$_4$ (ICN) in phosphate-free DMEM medium for 4 h. Following immunoprecipitation, samples were separated by SDS-PAGE, transferred to nitrocellulose membrane (Amersham Pharmacia Biotech), immunoblotted using monoclonal antibodies (202) (Sigma), and autoradiography was performed. c-Src

**RESULTS**

To determine whether α-Syn can be phosphorylated on tyrosine in a human cell line, the protein-tyrosine phosphatase (PTP) inhibitor pervanadate was utilized. Pervanadate, a complex of vanadate and hydrogen peroxide, is a competitive inhibitor of PTPs by pervanadate (Fig. 1A, B, and C). As illustrated in Fig. 1A and C, α-Syn is phosphorylated on tyrosine residues within 2 min of pervanadate treatment and increases incrementally over 20 min. Cells treated with sodium orthovanadate or hydrogen peroxide alone resulted in no change in α-Syn phosphorylation (data not shown). Tyrosine phosphorylation was confirmed by phosphoamino acid analysis (Fig. 1B). Phosphorylated serine but not threonine residues were also observed, confirming the results reported previously (1). Thus, α-Syn is phosphorylated on tyrosine in both a time- and dose-dependent manner following inhibition of PTPs by pervanadate (Fig. 1, A and C, data not shown).

To map the phosphorylated tyrosine residue(s), mutants were constructed by exchanging one or more of four tyrosine residues in α-Syn with either a phenyalanine or a stop codon (Fig. 2). These mutant constructs were transfected into HEK293T cells and treated for 20 min with pervanadate. Western analysis performed on immunoprecipitated α-Syn, using PY20 and 4G10 phosphotyrosine specific antibodies or Synuclein-1 antibodies indicates that the Y125F mutant construct is the only single tyrosine mutation that results in a significant effect, reducing tyrosine phosphorylation to ~5% of the wild-type control (Fig. 3). Tyrosine phosphorylation of the other single tyrosine mutation constructs was not significantly different from the wild-type construct. Phosphorylation of the Y133F mutant construct appeared to be greater than the wild-type; however, this difference was not statistically significant (Student’s t test). Interestingly, the Y133/136F double mutation resulted in an ~75% reduction in the tyrosine phosphorylation of α-Syn. Because neither single mutation alone results in a significant reduction in tyrosine phosphorylation, a possible explanation for this decrease is that this double mutation dis-
Fig. 2. Partial sequence of the human α-Syn protein illustrating the mutant constructs. All mutants were constructed by site-directed mutagenesis, according to manufacturer’s instructions (Stratagene) using the full-length wild-type cDNA as a template. Only amino acid sequences from 25 to 55, 85 to 90, and 120 to 140 are displayed. All constructs contain 140 amino acids except the Y125Stop and Y39F/Y125Stop constructs, which have only 124 amino acids. The solid lines are indicative of identical amino acids, whereas amino acid substitutions are indicated with single letter designations (X, stop codon). Tyrosine residues are highlighted in bold.

Fig. 3. α-Syn is phosphorylated primarily on tyrosine 125 in pervanadate-treated human HEK293T cells. A. HEK293T cells were transiently transfected with various FLAG-α-Syn constructs or empty vector (control lane) and treated with pervanadate for 20 min. Cells were harvested, α-Syn isolated, and Western analysis was performed as described in the legend to Fig. 1 except that instead of using duplicate membranes for normalization, the membranes were consecutively stripped and reprobed with 4G10 and Synuclein-1 antibodies respectively, following immunoblotting with the PY20 antibody. B. Values for each mutant construct were semi-quantitatively determined by normalizing the optical density values obtained by laser densitometry from immunoblots using PY20 antibody compared with values obtained using Synuclein-1 antibody. The mean values for each mutant construct, determined from multiple film exposures and from at least three separate experiments, are plotted as a percentage of the value obtained for the wild-type construct (± S.E., B).

rupts the interaction of α-Syn with a protein involved in phosphorylating tyrosine 125. Longer exposures reveal a very low level of phosphorylation of tyrosines 39, 133, and 136, but no phosphorylation in untransfected control cells or in Y39F/ Y125Stop-transfected cells, which do not contain any tyrosine residues. These data suggest that the majority of tyrosine phosphorylation of α-Syn in pervanadate-treated HEK293T cells occurs on tyrosine 125.

To determine the effects of the PD mutations on the tyrosine phosphorylation of α-Syn, mutant constructs were transfected into HEK293T cells and compared with the wild-type construct. Tyrosine phosphorylation of wild-type α-Syn in pervanadate-treated cells does not differ significantly from that of cells transfected with PD mutation constructs (A30P and A53T, data not shown). Additionally, the effect of mutating serines at positions 87 and 129 to alanine on tyrosine phosphorylation was determined, because these serines were shown previously to be phosphorylated (1). Phosphorylation of serines 87 and 129 is not necessary for phosphorylation of tyrosine 125 under the conditions tested (data not shown).

To help elucidate the PTK(s) that may be involved in the tyrosine phosphorylation observed with pervanadate, PP2, a selective inhibitor of the Src family of PTKs, was incubated with the cells as indicated in Fig. 4 (4). Western analysis revealed that these cells do express Src family members (data not shown). Wild-type α-Syn was transfected into HEK293T cells and pretreated with various concentrations of PP2 or a Me₂SO-vehicle control for 1 h prior to treatment with pervanadate. Western analysis was then performed on immunoprecipitated α-Syn using the PY20 and Synuclein-1 antibodies, and mean values for each PP2 treatment group were plotted as a percentage of the vehicle-treated control (Fig. 4). PP2 inhibits the pervanadate-induced tyrosine phosphorylation of α-Syn with an EC₅₀ of ~1 μM, a concentration reported to inhibit effectively the function of Src family PTKs in human T-cells, suggesting that Src family PTKs are able to phosphorylate α-Syn expressed in HEK293T cells (4).

To determine more directly whether Src family members can phosphorylate α-Syn in these cells, cotransfection experiments were performed utilizing c-Src and Fyn expression vectors as depicted in Fig. 5A. Additionally, a mutant c-Src (K295M), which inactivates the kinase by disrupting its phosphotransfer activity, and Rlk, a nonreceptor PTK whose expression is limited to T-cells and mast cells, were utilized as controls (24–26). Wild-type (Fig. 5A) and various mutant α-Syn constructs (Fig. 5B) were cotransfected into HEK293T cells along with the PTK expression constructs. Western analysis was then performed on immunoprecipitated α-Syn using the PY20 and FLAG antibodies (Fig. 5A, panels 1 and 2; B), or on total lysates using SRC2 and GFP antibodies (Fig. 5A, panels 3 and 4). As illustrated in Fig. 5A, c-Src and Fyn, but not the mutant c-Src (K295M) or Rlk-GFP, cotransfected with FLAG-α-Syn results in increased tyrosine phosphorylation of α-Syn. Western analysis of total lysates confirmed not only that c-Src, Fyn, and Rlk-GFP are expressed, but also that these PTKs are active in these cells, because large increases in tyrosine phosphorylation is observed in cells transfected with these PTKs compared with untransfected and mutant c-Src (K295M)-transfected cells (data not shown). Although Rlk is active in these cells, it does not phosphorylate α-Syn under these conditions, indicating that not all cotransfected PTKs are capable of phosphorylating α-Syn.

As observed in Fig. 3, with pervanadate, c-Src cotransfected with the Y125F α-Syn mutant construct also results in a significant reduction in tyrosine phosphorylation as compared with the wild-type construct, whereas other single amino acid
changes do not differ significantly (Fig. 5B). This indicates that tyrosine 125 is also the major tyrosine phosphorylation site in c-Src-cotransfected cells. Src family PTKs could be phosphorylating α-Syn directly, or indirectly by acting through Src-activated pathways involving other kinase(s). To help distinguish between these two possibilities, in vitro kinase assays using purified c-Src, Fyn, and α-Syn were performed. α-Syn (250 or 500 ng) was incubated with 0.4 or 2 units of p59 fyn or p60c-src in kinase buffer containing [γ-32P]ATP. Enolase (250 ng) was used as a positive control, because it is a substrate for c-Src and Fyn (22, 27). The reactions were separated by SDS-PAGE, and autoradiography was performed. Results using 2 units of kinase and 500 ng of α-Syn are shown in Fig. 5C. α-Syn was phosphorylated in vitro by both Fyn (panel 1) and c-Src (panel 2), whereas no phosphorylation was observed in experiments where α-Syn was incubated without PTKs or if bovine serum albumin was provided as a substrate (data not shown). Fyn and c-Src could also phosphorylate α-Syn at lower concentrations of kinase (0.4 units) and substrate (250 ng) than those shown in Fig. 5C (data not shown).

To compare the relative ability of Fyn and c-Src to phosphorylate α-Syn, the ratio of the α-Syn signal versus the enolase signal for each kinase was compared. The ability of Fyn and c-Src to phosphorylate enolase under the conditions tested was approximately the same per unit of kinase, although not readily apparent because different exposure times are shown in panels 1 and 2 in Fig. 5C. Relative to enolase, Fyn phosphorylated α-Syn ∼120 times better than c-Src as determined by laser densitometry of multiple exposures.

**DISCUSSION**

It is of great interest to determine the specific function(s) of α-Syn because of its potential importance in the pathogenesis of PD. Studying post-translational modifications, such as phosphorylation, can be very useful in gaining insight into protein function. α-Syn is phosphorylated on serine residues; however, tyrosine phosphorylation of α-Syn had not been established (1, 2). A protein alignment of α-, β-, and γ-Syns revealed that all four of the tyrosine residues of α-Syn, located at positions 39, 125, 133, and 136, are conserved in all identified orthologs and β-Syn paralogs, whereas only the tyrosine at position 39 is conserved in γ-Syn, suggesting that these residues may be functionally important (3).

The data presented in this manuscript indicate that α-Syn is phosphorylated on tyrosine in response to pervanadate inhibition of PTPs. This tyrosine phosphorylation occurs primarily on tyrosine 125 and is inhibited by PP2, implicating the involvement of the Src family of PTKs. Additionally, specific members of the Src family of PTKs, c-Src and Fyn, phosphorylate α-Syn directly in cotransfection experiments and in *in vitro* kinase assays using purified kinases. The data indicate that α-Syn can be phosphorylated by the Src family of PTKs and suggest that α-Syn is a possible substrate for Src family members in the brain; although, additional experiments are necessary to determine the PTK(s) that phosphorylate α-Syn in vivo.

Sequences flanking tyrosine 125, as depicted in Fig. 2, closely resemble those of the optimal substrate sequences for PTKs determined using an oriented peptide library technique (28). For example, this tyrosine 125 site (DNEAYEMP) is similar to the c-Src optimal substrate sequence which was determined to be DEEIY(G/E)EF. The amino acids flanking tyrosine 125 at positions −4, −2, and +1 are identical to this optimal substrate sequence, whereas the alanine at position −1 is similar to the isoleucine in the ideal sequence in that they are both hydrophobic residues. Although the methionine and proline residues at positions +2 and +3, relative to the phosphorylated tyrosine, are not optimal substrate residues for c-Src phosphorylation, they are optimal residues for phosphorylation by other PTKs (28). Thus, the tyrosine 125 site is consistent with optimal substrate sequences of PTKs including c-Src.

It is difficult to speculate on the functional consequences of tyrosine phosphorylation of α-Syn, because its normal function has not been elucidated definitively. α-Syn is primarily a soluble protein expressed in presynaptic neurons, but is also loosely associated with synaptic vesicles (29). α-Syn is also implicated in regulating a form of dopamine plasticity in an α-Syn knockout mouse model, and maintenance of the distal pool of synaptic vesicles in primary hippocampal cultures (30, 31). Covalent modification, such as phosphorylation, is a likely candidate for regulation of α-Syn at the synapse and could be important in modulating its function. Tyrosine phosphorylation occurs in synaptic vesicles and is important for regulating synaptic function in the brain (15, 32). For example, tyrosine kinase inhibitors have been shown to block long-term potentiation in the hippocampus, and increased tyrosine phosphorylation in the squid giant synapse modulates synaptic transmission by increasing calcium currents, both implicating PTKs in synaptic plasticity (16, 33). Fyn and c-Src are also thought to be involved in spatial learning and synaptic plasticity (34–36). For example, Fyn knockout mice exhibit an impairment in memory and learning, which is thought to be caused by alterations in long-term potentiation (35).

The Src family of nonreceptor PTKs is expressed ubiquitously with increased expression in neurons and hematopoietic cells (37). The possibility that members of the Src family of PTK(s) may be involved in the modification of α-Syn is intriguing because of overlap in brain region specific expression and subcellular localization between c-Src and α-Syn. Both proteins are expressed highly in the brain and also have overlapping expression in various regions, for example, in the hippocampus (3, 11, 36). Subcellularly, both of these proteins are loosely associated with synaptic vesicles in presynaptic neurons (12, 38). c-Src has also been shown to be active at the synapse by...
phosphorylating synaptic vesicle proteins such as synaptophy- 
sin and synaptotagmin (38).

The apparent 120-fold-increased phosphorylation of α-Syn by Fyn versus c-Src, relative to enolase, raises two interesting possibilities that could account for this increase (Fig. 5C). The first is that α-Syn is a more preferred substrate for Fyn than for c-Src. The second is that some enhancing factor is copurifying with Fyn because it was purified by sequential chromatography from a membrane fraction of bovine thymus, whereas recombinant human c-Src was purified from baculovirus. We believe that the latter explanation is more likely because no significant difference in the relative amount of tyrosine phosphorylation of α-Syn exists in cotransfection experiments comparing c-Src and Fyn despite similar protein expression (Fig. 5A, panels 1–3). This hypothesized factor enhances greatly the in vitro activity of Fyn for α-Syn, but not enolase, and therefore is specific for α-Syn. To prove this hypothesis Fyn and c-Src must be isolated by an equivalent means, allowing for direct comparison and ultimately one must isolate and identify this “enhancing factor”.

Although the functional consequences of phosphorylation of the tyrosine 125 residue of α-Syn remain to be elucidated, it could regulate its ability to bind synaptic vesicles and/or be involved in regulating protein/protein interactions. It has been reported that phosphorylation of the serine 129 residue of α-Syn results in a reduction in binding to phospholipid containing liposomes (2). Additionally, phosphorylation regulates the association of the synaptic vesicle protein, Synapsin I to synaptic vesicles (39). Similarly, tyrosine phosphorylation may allow for the dynamic regulation of α-Syn binding to synaptic vesicles. Interestingly, Synapsin I has also been shown to interact with c-Src and reportedly increases c-Src tyrosine kinase activity (40).

Recently, the microtubule-associated protein Tau was identified as a binding partner of α-Syn (41). Colocalization of Tau and α-Syn was also demonstrated in axons. Tau associates with the C-terminal region (residues 89–140) of α-Syn, through its microtubule binding domain. Jensen and co-workers (41) hypothesized that this interaction between α-Syn and Tau could link synaptic vesicles with microtubules. Tau has also been shown to both colocalize and interact directly with the Src PTK family member, Fyn (42). Hypothetically, Tau could bring Src PTK family members, such as Fyn, into close proximity to α-Syn, thereby enhancing the activity of these kinases for α-Syn. It is also conceivable that the association of α-Syn and Tau could be regulated by phosphorylation of tyrosine 125, and that in certain populations of synaptic vesicles could regulate their attachment to microtubules. This could allow α-Syn to bind and releaseTau in a phosphorylation-dependent manner, and thereby contribute to the maintenance of the distal pool of synaptic vesicles.

Acknowledgments—We thank Drs. Virginia Lee and Susan Reuter for the HEK293 cells stably transfected with human α-Syn and Drs. Melanie Hartsough, Nelson Cole, and Michael Czar for helpful discussions.

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J. Biol. Chem. 2001, 276:3879-3884.
doi: 10.1074/jbc.M010316200 originally published online November 14, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M010316200

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