Efficient Modification of Alpha-Synuclein Serine 129 by Protein Kinase CK1 Requires Phosphorylation of Tyrosine 125 as a Priming Event

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ABSTRACT: S129-phosphorylated alpha-synuclein (α-syn) is abundantly found in Lewy-body inclusions of Parkinson’s disease patients. Residues neighboring S129 include the α-syn tyrosine phosphorylation sites Y125, Y133, and Y136. Here, we use time-resolved NMR spectroscopy to delineate atomic resolution insights into the modification behaviors of different serine and tyrosine kinases targeting these sites and show that Y125 phosphorylation constitutes a necessary priming event for the efficient modification of S129 by CK1, both in reconstituted kinase reactions and mammalian cell lysates. These results suggest that α-syn Y125 phosphorylation augments S129 modification under physiological in vivo conditions.

KEYWORDS: Alpha-synuclein, phosphorylation, kinetics, casein kinase 1, Parkinson’s disease, NMR

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ggregation of human α-syn into amyloid-rich Lewy bodies constitutes a hallmark of Parkinson’s disease (PD) and other related synucleinopathies.1 Whereas 90% of aggregated α-syn in neuronal inclusions is phosphorylated at S129, less than 4% of the soluble protein is modified,2 which supports a possible link between α-syn S129 phosphorylation, protein aggregation, and PD pathogenesis.3,4 In intact neurons, S129 phosphorylation is thought to modulate dopamine uptake and to mediate the Rab8a blocking synaptic vesicle trafficking.5,6 Given this importance of α-syn S129 phosphorylation, several kinases have been identified that establish this modification in vitro and in vivo, including members of the Polo-like kinase family (Plk’s) and protein kinases CK1 and CK2 (formerly known as casein kinases I and II).7–12

In comparison to the modification of S129, phosphorylation of α-syn tyrosines is less well explored. Y125-phosphorylated α-syn is found in Lewy bodies,13 although no clear correlation between this modification and disease onset, or progression has been established.14 Possible roles of Y133 and Y136 phosphorylation are equally poorly understood.14,15 By contrast, phosphorylation of α-syn Y39 contributes to cytotoxicity by impairing the cellular clearance of α-syn.16 All four tyrosines of α-syn, that is, Y39, Y125, Y133, and Y136, are targeted by members of the Sarcoma (Src) family of kinases (SFKs) both in vitro and in vivo,17 with Fyn kinase being the most effective enzyme.18 Fyn preferentially targets Y125, whereas Y39, Y133, and Y136 constitute secondary phosphorylation sites.17 Other enzymes, such as the acidophilic tyrosine kinase Syk, are reported to phosphorylate Y125 and, to a lesser extent, Y133 and Y136,19 whereas Y39 is thought to be the preferred in vivo substrate site for Abl kinases.16

α-Syn S129 and tyrosines Y125, Y133, and Y136 are in close proximity, which raises the question whether crosstalk between these phosphorylation sites exists. Recent evidence suggests that phosphorylated Y125 and S129 occur together in Lewy bodies;13 however, serine-tyrosine phosphorylation crosstalk has not been investigated before. The closeness of these C-terminal modification sites complicates any such analysis, especially when classical approaches of mutagenesis and immunodetection, or mass spectrometry are employed. Kinase activities may be lost upon sequence changes of substrate sites and antibody fidelity may be compromised on altered epitopes.20 Similarly, the phosphorylation states of neighboring substrate sites are difficult to assess with mass spectrometry, because uniquely modified, proteolytic peptide fragments are not generated easily. To overcome these problems, we employed time-resolved high-resolution NMR spectroscopy to map and monitor quantitatively α-syn serine-tyrosine phosphorylation in reconstituted kinase reactions and mammalian cell lysates.21,22 Our data show that different tyrosine kinases display clear substrate site preferences on α-syn and that phosphorylation of Y125 constitutes a necessary priming event for the efficient modification of S129 by CK1. These results suggest that modification of Y125 may increase the phosphorylation levels of S129 by the ubiquitously acting kinase CK1, thus strengthening the link between Y125–S129 phosphorylation and PD pathology.
RESULTS AND DISCUSSION

We tested an extended set of receptor (RTKs) and nonreceptor tyrosine kinases (non-RTKs) for their ability to phosphorylate N-terminally acetylated α-syn. After incubating 25 μM (3.75 nmol) 15N isotope-labeled α-syn with 250 enzymatic units (U) of each enzyme for ~12 h, we recorded two-dimensional (2D) 1H−15N correlation spectra to determine the individual phosphorylation levels of Y39, Y125, Y133, and Y136 (Figure 1a and Supporting Information Figure 1a). Our results showed that all tested kinases phosphorylated α-syn to different extents and that modification by non-RTKs was generally more efficient than phosphorylation by RTKs (Figure 1b). Members of the Src family of kinases (SFKs), including Yes, Src, Fyn, and Lyn, preferentially targeted Y125, whereas Y136 was the preferred substrate site of focal adhesion kinase 1 (FAK1), but not of FAK2. Phosphorylation of Y125 with Fyn and Syk reached 100%, indicating that both kinases targeted this site with high efficiency, in line with previous reports. Compared to other α-syn tyrrosines, Y39 phosphorylation was least efficient and only few kinases, namely Axl, Ron, and Abl-1 and -2, modified this site specifically. Interestingly, active forms of Abl are up-regulated in response to dopamine-derived neurotoxins and in PD patients’ brains. These data established that different tyrosine kinases target different α-syn sites in vitro. While some of them have clear implications in α-syn’s biology, as outlined previously, the physiological relevance of other kinases, especially with regard to possible roles in PD, remains to be determined. Here, our results may provide initial points of reference for future functional studies. In addition, information about tyrosine kinase specificities and α-syn site preferences may be used to generate uniquely modified versions of the protein for biochemical analyses.

Figure 1. In vitro phosphorylation of α-syn tyrosines. (a) Schematic representation of α-syn phosphorylation by Fyn (top) and selected view of the 2D 1H−15N SOFAST-HMQC spectra of N-terminally acetylated, but otherwise unmodified α-syn (black) and of tyrosine-phosphorylated α-syn (blue) (bottom). Labels indicate the chemical shift changes characteristic for the individual tyrosine phosphorylation sites. (b) Phosphorylation of α-syn by different RTKs and non-RTKs (250 U). Percentages of site-specific phosphorylation levels were quantified based on NMR cross-peak intensities after ~15 h of kinase incubation. Kinases are ordered according to their evolutionary distances. Experiments were performed on 25 μM α-syn samples (150 μL) in NMR buffer at 25 °C and pH 7.0.

In a next step, we monitored α-syn tyrosine phosphorylation in a time-resolved fashion by recording consecutive sets of 2D 1H−15N correlation experiments of reconstituted kinase reactions proceeding directly inside the NMR spectrometer (Figure 2a). By plotting the changes of individual α-syn peak intensities against the reaction time, we established that Fyn phosphorylated Y125 faster than Y133, Y136, and Y39, whereas Syk phosphorylated Y125 and Y136 with comparable rates, in agreement with our previous results. Given that S129 is four residues away from Y125 and Y133, we asked whether modification crosstalk between these sites existed, and whether S129 modification affected the rates of tyrosine phosphorylation by Fyn and Syk. First, we prephosphorylated 15N isotope-labeled, N-terminally acetylated α-syn at S129 with Plk3 (Supporting Information Figure 1b). Next, we reacted unmodified and S129-modified α-syn with 250 U of Fyn and Syk in time-resolved reaction setups to measure tyrosine phosphorylation rates (Figure 2a). For the reaction with Fyn, we found no significant differences to unmodified α-syn, whereas Syk displayed a slight decrease in the rate of Y125 phosphorylation and a slight increase in the Y133 modification efficiency. Overall, S129 phosphorylation did not exert pronounced effects on the substrate site preferences and the general activities of both enzymes, in agreement with previous findings. We also analyzed whether reciprocal tyrosine prephosphorylation influenced the modification of S129 by Plk3, CK1, and CK2, three kinases reported to phosphorylate this site. We set up Plk3 reactions (250 U) with unmodified and fully tyrosine premodified α-syn substrates and found very similar S129 phosphorylation rates (Figure 2b). We did not detect S129 phosphorylation of unmodified, or tyrosine-phosphorylated α-syn with 250–2000 U of CK2 (Supporting Information Figure 2a), in agreement with recent studies showing that α-syn is a poor CK2 substrate in vitro.

Although CK1 has been used previously to phosphorylate α-
We did not observe phosphorylation of unmodified α-syn with 250 U of CK1 under our experimental conditions (Figure 2c, left). This discrepancy likely arose from the relatively low enzyme amounts that we used in our assay. Indeed, when we increased CK1 concentration to 2000 U, we detected 10% of S87 and S129 phosphorylation after 24 h of incubation (Suppl. Figure 2b), suggesting that CK1 phosphorylated unmodified α-syn with low efficiency. In stark contrast, we detected virtually complete modification of S129 when we reacted tyrosine-phosphorylated α-syn with 250 U of CK1 (Figure 2c), from which we inferred that modified tyrosines greatly enhanced the CK1-mediated modification of S129.

Because CK1 typically requires “priming” via a phosphorylated residue at the −3 position for efficient catalysis, we asked whether phosphorylated Y125 (−4 position) functioned as such a priming site for CK1-mediated phosphorylation of S129. To answer this question, we individually replaced either Y133 or Y125 with nonmodifiable phenylalanines, leaving the respective other tyrosine intact, and prephosphorylated both mutants with Fyn (Figure 3a). Upon subsequent incubation with CK1 (250 U), we observed efficient S129 phosphorylation of the Y133F mutant, containing the intact phospho-Y125 site, but not of the Y125F mutant with intact phospho-Y133. These results suggested that Y125 phosphorylation functions as the priming event that enables efficient modification of S129 by CK1 in vitro.

Next, we asked whether Y125 phosphorylation constituted a similarly important priming event for the phosphorylation of S129 by cellular CK1. In a first instance, we tested lysates from cultured mammalian A2780, K562, B65, SK-N-SH, SH-SY5Y, and RCSN-3 cells for the presence of endogenous CK1, and members of the Plk family of kinases (Figure 3b). Western blotting showed that CK1, Plk2, and partially also Plk3 are abundantly present in these cells. Upon the addition of 15N isotope-labeled, unmodified α-syn to cell lysates, we did not detect S129 phosphorylation by NMR spectroscopy (data not shown). To investigate whether greater amounts of cellular kinases were needed to phosphorylate S129, we transiently overexpressed Plk3 in human A2780 cells.
phospho-peptides containing a modiﬁcation in a compelling manner. Previous results indicated that α-syn is abundantly expressed in many different cell types, including those of the brain.31,32 In addition, colocalization of α-syn and α-syn aggregates in proteinaceous brain inclusions of transgenic α-syn under these experimental conditions. Next, we added tyrosine prephosphorylated α-syn to native cell lysates and recorded in situ NMR experiments on the resulting mixtures (Figure 3c and Supporting Information Figure 4). This time, we detected efﬁcient de novo phosphorylation of S129 in all lysates, presumably by endogenous CK1. To test this idea, we performed similar reactions in the presence of increasing amounts of D4476, a CK1-speciﬁc kinase inhibitor.29 In support of our hypothesis, CK1 inhibition progressively diminished S129 phosphorylation in native A2780 cell lysates, which suggested that priming via Y125 phosphorylation was also required for the efﬁcient phosphorylation of S129 by cellular CK1.

From a mechanistic point of view, efﬁcient CK1 priming via a phospho-tyrosine residue at the −4 position expands the known repertoire of −3 phospho-serine/threonine priming sites in a compelling manner. Previous results indicated that phospho-peptides containing a modiﬁed serine residue at the −4 position can only stimulate weak CK1 activity,28 whereas phospho-tyrosine priming at the canonical −3 site does not lead to measurable CK1 activity.25 In the context of our results, this suggests that the CK1-priming potential of phospho-tyrosines at the −4 position may be similar to the one of phospho-serines/threonines at the −3 site. Whether other examples of CK1 substrates with phospho-tyrosine priming sites exist remains to be determined. In the case of α-syn, phospho-Y125 mediated modiﬁcation of S129 by CK1 may be of particular interest because CK1 is constitutively active and abundantly expressed in many different cell types, including those of the brain.31,32 In addition, colocalization of CK1 and α-syn aggregates in proteinaceous brain inclusions of transgenic α-syn mice and in post mortem structures of patients with Lewy-body dementia and PD has been established.26 Thus, transitions to the S129-phosphorylated form of α-syn may also result as a consequence of Y125 phosphorylation, a notion that has not yet received much attention. The presence of four post-translational phosphorylation sites within a narrow stretch of amino acids in the C-terminus of α-syn indicates a hot spot for regulated interactions. Indeed, many of the previously identiﬁed α-syn binding partners target this region, including the dopamine transporter (DAT),33 the GTPase Rab8a, and others. Future investigations will determine how different serine and/or tyrosine phosphorylation states modulate these interactions.

**METHODS**

**NMR Spectroscopy.** End point and time-resolved NMR spectra of phosphorylation reactions were recorded on 600 and 750 MHz Bruker Avance spectrometers, equipped with cryogenically cooled triple resonance 1H/13C/15N TCI probes, on α-syn samples dissolved in NMR buffer at pH 7.0. 2D 1H−15N SOFAST-HMQC experiments were recorded at 25 °C. Spectra were acquired with 1024 (H) and 256 (15N) complex points, 44 scans, and a recycling delay of 60 ms (∼30 min acquisition time). Pulse sequence and its parameters were used as described previously.21 To delineate phosphorylation rates, we measured cross-peak intensities and/or signal volumes of unambiguously assigned, well-resolved NMR cross-peaks reporting on the individual phosphorylation states of residues Y39, Y125, Y133, Y136, and S129 in the different time-resolved NMR spectra, as reported previously.22 These were N122 and D121 for Y125 phosphorylation; E139 and A140 for Y136 phosphorylation; Y133 for Y133 phosphorylation; L38, V40, and G41 for Y39 phosphorylation; and L38, V40, and G41 for Y39 phosphorylation.

**In Vitro Phosphorylation Reactions.** Tyrosine kinases were purchased from SignalChem unless stated otherwise. CK1-δ and CK2 were purchased from New England Biolabs. Fyn kinase was also obtained recombinantly, as a kind gift of Benno Kurokpa, FMP Berlin, and we determined that 10 μL of this preparation corresponded to 250 U of commercial Fyn (SignalChem). Plk3 was purchased from Millipore. Unless otherwise speciﬁed, in vitro phosphorylation reactions were carried out in duplicates at 25 °C with 250 U (1 U = 1 pmol/min) of the respective kinases and 2.5 μM of α-syn in NMR buffer at pH 7.0, supplemented with 1 mM ATP (Fermentas) and 5 mM MgCl2, MnCl2, or DTT was also added in some reactions according to suppliers’ instructions. Final reaction volumes were 150 μL in all cases.

**Transient Plk3 Overexpression.** Adherent A2780 cells were transfected with either pEGFP-C1 (mock, Clontech) or PLK3 1-344-pEGFP, encoding the kinase domain of human Plk3 fused to GFP (Addgene plasmid 23266),34 by using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. At 22 h after transfection, cells were detached and collected by mild trypsin treatment, washed twice in PBS, and resuspended in NMR buffer at pH 7.0 containing 1X protease inhibitors (complete EDTA-free, Roche) and 1X phosphatase inhibitor cocktails 2 and 3 (Sigma). Cells were centrifuged for 5 min at 1000 rpm, and the supernatant was discarded. The wet cell pellet was lysed by ﬁve rounds of freeze—thaw using a dry ice/ethanol bath, after which the soluble fractions were obtained by a 10 min spin at 16 000g at 4 °C. Protein concentrations were determined using a Bradford assay (Bio-Rad). Extracts were used immediately after preparation or aliquoted and snap-frozen in liquid nitrogen and stored at −80 °C until further use. Freeze—thaw cycles were avoided in order to preserve enzymatic activities.35 Samples for Western blotting were boiled for 5 min in 1X Laemmli buffer (Bio-Rad). Mammalian cell lines were as follows: A2780 (human, ovarian, Sigma-Aldrich), K562 (human, leukemia, DSMZ), B65 (rat, neuroblastoma, Sigma-Aldrich), SH-SY5Y (human, neuroblastoma, Sigma-Aldrich), and SK-N-SH (human, neuroblastoma, Sigma-Aldrich). For phosphorylation reactions, extracts were adjusted to the desired total protein concentration in NMR buffer supplemented with phosphate inhibitor cocktails 2 and 3 (Sigma). 1 mM ATP, and 5 mM MgCl2 (final reaction volume, 150 μL). Reactions were carried out in duplicates at 25 °C by adding 25 μM of isotope-labeled α-syn directly into the different cell lysates and immediately starting the NMR experiments. For the CK1 inhibition experiment, we added the indicated amounts of the CK1 inhibitor D4476 (Millipore) to A2780 cell lysates as previously reported.22

**ASSOCIATED CONTENT**

**Supporting Information**

Experimental details for NMR resonance assignments of tyrosine phosphorylated α-syn and immunoblotting. NMR spectra of unmodiﬁed and serine-tyrosine phosphorylated α-syn, phosphorylation of S129 by CK1 and CK2, de novo S129 phosphorylation in lysates of A2780 cells transiently overexpressing Plk3 or supplemented with a CK1 inhibitor. This
material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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