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α-Synuclein phosphorylation as a therapeutic target in Parkinson's disease

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Abstract

Phosphorylation is a key post-translational modification necessary for normal cellular signaling and, therefore, lies at the heart of cellular function. In neurodegenerative disorders, abnormal hyperphosphorylation of pathogenic proteins is a common phenomenon that contributes in important ways to the disease process. A prototypical protein that is hyperphosphorylated in the brain is α-synuclein (α-syn) – found in Lewy bodies and Lewy neurites – the pathological hallmarks of Parkinson's disease (PD) and other α-synucleinopathies. The genetic linkage of α-syn to PD as well as its pathological association in both genetic and sporadic cases have made it the primary protein of interest. In understanding how α-syn dysfunction occurs, increasing focus is being placed on its abnormal aggregation and the contribution of phosphorylation to this process. Studies of both the kinases and phosphatases that regulate α-syn phosphorylation are beginning to reveal the roles of this post-translational modification in disease pathogenesis. Modulation of α-syn phosphorylation may ultimately prove to be a viable strategy for disease-modifying therapeutic interventions. In this review, we explore mechanisms related to α-syn phosphorylation, its biophysical and functional consequences, and its role in neurodegeneration.

Keywords: aggregation; casein kinase; GRK; LRRK2; PLK; PP2A.

Introduction: α-synuclein (α-syn) and disease

α-Syn is a protein that is highly abundant in the brain (Iwai et al., 1995). Although its physiological functions remain to be fully elucidated, its dysfunction has been the central focus for a number of neurodegenerative disorders known as α-synucleinopathies, which include Parkinson's disease (PD), diffuse Lewy body disease (DLB), and multiple system atrophy (MSA). These disorders are characterized by abnormal aggregation of α-syn in pathological hallmark inclusions in neurons known as Lewy bodies and Lewy neurites in PD and DLB, and as glial cytoplasmic inclusions (GCIs) in oligodendroglia in MSA (Dickson et al., 1999). This pathological connection has placed α-syn at the forefront in investigations of molecular mechanisms of disease pathogenesis. The connection has been further strengthened by genetic linkage. Rare mutations in the α-syn locus give rise to PD with pathological and clinical characteristics of sporadic forms of the disease. Duplication and triplication of the gene encoding α-syn have been found in a number of families with dominantly inherited PD, often associated with dementia (Singleton et al., 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004). In these patients, α-syn aggregation is amplified and earlier symptom onset is observed. This acceleration of pathology and functional decline is indicative of α-syn's role in neurodegeneration. Furthermore, three point mutations have been identified, namely, A53T (Polymeropoulos et al., 1997), A30P (Kruger et al., 1998), and E46K (Zarranz et al., 2004), which also lead to early-onset forms of PD. Characterization of the functional consequences of these mutations has indicated that they promote α-syn aggregation (Conway et al., 1998), further linking this dysfunctional property to disease. Together, these genetic and pathological clues have implicated α-syn as a central player in disease pathogenesis. Therefore, understanding its mechanistic connections at the biochemical and functional levels is important to elucidating the pathogenesis of α-synucleinopathies and for developing disease-modifying therapeutics.

Biochemistry of α-syn

α-Syn is the only member of a family of synucleins that has been linked to disease (Lavedan, 1998). It is a 14 kDa protein with three domains: an α helical N-terminal domain, a central hydrophobic non-Aβ component (NAC) domain that is critical for aggregation, and a C-terminal intrinsically disordered domain that is proline rich and highly acidic (Figure 1). The N-terminus contains six α helical repeats of 11 residues with variations of the conserved central sequence KTKEGV; this region is thought to be important in anchoring and localizing α-syn (Bartels et al., 2010). The isolated NAC domain forms amyloid structures, and small deletions within this domain can dramatically reduce the propensity of α-syn to aggregate (Rivers et al., 2008). The

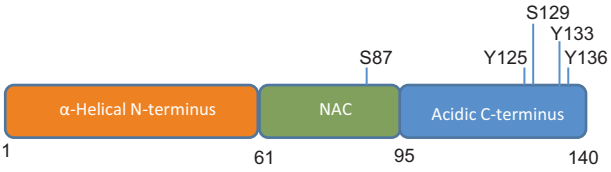

Figure 1 Structure and phosphorylation sites of α-syn.
The protein has three domains – an N-terminal α-helical domain; a central, hydrophobic, NAC domain; and an intrinsically disordered, acidic, C-terminal domain. The experimentally identified phosphorylation sites are indicated.

C-terminal domain also appears to suppress α-syn aggregation; C-terminal truncations have been associated with α-syn aggregates in human brains as well as in animal models (Li et al., 2005), and *in vitro* studies have demonstrated that such species aggregate more rapidly than full-length α-syn (Rochet et al., 2000; Murray et al., 2003).

Phosphorylation of α-syn

α-Syn is subject to a number of post-translational modifications (reviewed in Oueslati et al., 2010). Of these, the effects of phosphorylation are the most thoroughly studied, although other modifications including ubiquitinylation and truncation may also have functional consequences. α-Syn is subject to both serine and tyrosine phosphorylation, with phosphorylation observed experimentally at Ser87, Ser129, Tyr125, Tyr133, and Tyr136 (Okochi et al., 2000; Pronin et al., 2000; Fujiwara et al., 2002; Negro et al., 2002). *In vivo* phosphorylation at Ser87, Ser129, and Tyr125 have been identified; therefore, the major focus has been on these phosphorylation sites. Postmortem human brain studies have shown that α-syn in Lewy bodies and GCIs is hyperphosphorylated at Ser129 (Fujiwara et al., 2002; Anderson et al., 2006). Phosphorylation at this site is also observed in pre-Lewy body stages – in Lewy threads, grains and axons, apparently progressing with advancing disease (Saito et al., 2003). Additionally, in α-syn transgenic mice (Wakamatsu et al., 2007; Schell et al., 2009; Lee et al., 2011b) as well as in primates overexpressing α-syn through a viral vector in the ventral midbrain (Eslamboli et al., 2007), Ser129 phosphorylation is evident. Together, data from postmortem human studies of α-synucleinopathies and animal models indicate that phosphorylation is strongly linked to disease progression.

Regulation of α-syn phosphorylation – kinases

The enzymes that modulate α-syn phosphorylation have been of major interest, in part as they may provide therapeutic targets. Studies across *in vitro* and *in vivo* systems have most convincingly demonstrated that Polo-like kinases (PLKs) phosphorylate at Ser129 (Inglis et al., 2009; Mbefo et al., 2010), although *in vitro* and cellular studies have demonstrated that Casein kinases 1 and 2 (Okochi et al., 2000) and G-protein receptor coupled kinases 1, 2, 5, and 6 (Pronin et al., 2000) and perhaps even Leucine-rich repeat kinase 2 (LRRK2) (Qing et al., 2009) can also phosphorylate at this residue. Tyrosine phosphorylation can be achieved by Src, Lyn, Frg, Syk, and Fyn kinases. Such redundancy is common to a number of highly phosphorylated proteins such as tau, which is subject to phosphorylation by a multitude of different kinases (Dolan and Johnson, 2010). Redundancy is likely a cellular mechanism to ensure that phosphorylation can be regulated at multiple steps and cannot go awry with limitations in specific signaling pathways. However, this is counterproductive for therapeutic strategies as inhibition of multiple kinases may be required to reduce phosphorylation. Nonetheless, each of these kinases can provide important understanding of the roles of phosphorylation of α-syn in disease pathogenesis and may be manipulated for disease modification.

Polo-like kinases

PLKs are a family of kinases which are critically involved in cell cycle progression and cellular proliferation (Barr et al., 2004). There are four PLK isoforms (PLK 1–4), with the common feature of a polo box domain that targets them to specific cellular locales (Lee et al., 1998). PLK2 has been demonstrated to most strongly phosphorylate α-syn at Ser129 (Inglis et al., 2009; Mbefo et al., 2010), with PLK3 and PLK1 potently contributing (Inglis et al., 2009; Mbefo et al., 2010). The N-terminus of α-syn is the critical domain for interaction with PLKs to allow its phosphorylation at Ser129 in the C-terminus. Interestingly, the effect of PLK2 is specific to α-syn with no phosphorylation of β-syn. It remains unclear why there is such specificity as these are highly homologous proteins, and the major variable region (10 amino acids in NAC) does not appear to influence PLK action (Mbefo et al., 2010). PLKs do not phosphorylate γ-syn, whose sequence around the equivalent serine residue is distinct from other synuclein isoforms. PLKs can act on both monomeric α-syn and α-syn fibrils, therefore having multiple stages of α-syn aggregation at which they could influence its phosphorylation status (Mbefo et al., 2010; Waxman and Giasson, 2011).

Casein kinases (CK1 and CK2)

Casein kinases are ubiquitous enzymes involved in a diverse array of processes including regulation of Wnt signaling (Price, 2006), control of circadian rhythm (Lee et al., 2011a), and cell cycle control (Behrend et al., 2000). CK1 and CK2 have been demonstrated to phosphorylate α-syn at Ser129, but only CK1 appears to phosphorylate Ser87 *in vitro* (Okochi et al., 2000; Waxman and Giasson, 2008). CK2 has been shown to phosphorylate Ser129 in cells and neuronal tissue, co-localizes with α-syn in the halo of Lewy bodies in PD (Ryu et al., 2008), and both CK2 and phosphorylated α-syn are associated with insoluble aggregates in diseased brains (Ishii et al., 2007). CK2 appears to only be able to phosphorylate soluble α-syn, whereas PLKs can also

phosphorylate insoluble aggregated species (Waxman and Giasson, 2011).

G-protein-coupled receptor kinase (GRK)

GRKs are important kinases involved in desensitization of G-protein-coupled receptors (GPCRs) (Pitcher et al., 1998). In addition to their roles in regulating GPCR signaling, they can phosphorylate additional substrates including α-syn (Pronin et al., 2000). The specific relevant isoforms are unclear as GRK2 and GRK5 phosphorylate α-syn *in vitro* and with overexpression in cells (Pronin et al., 2000), while in knockdown experiments, GRK2 and GRK5 appear not to modulate α-syn phosphorylation, but GRK3 and GRK6 have significant effects (Sakamoto et al., 2009).

Leucine-rich repeat kinase 2 (LRRK2)

LRRK2 is a kinase that has strong genetic linkage to dominantly inherited PD (Kumari and Tan, 2009). Clinically identified mutations in LRRK2 generally result in its increased kinase activity (Anand and Braithwaite, 2009). Therefore, mechanistically understanding the pathways in which it is involved is of critical importance for PD. Multiple studies have indicated potential linkage between α-syn and LRRK2 (Alegre-Abarrategui et al., 2008; Lin et al., 2009; Carballo-Carbajal et al., 2010), although there is only limited data of a direct phosphorylation (Qing et al., 2009) which has not been replicated in other studies or in cellular or animal models of manipulating these proteins. Most likely, there are indirect interactions between LRRK2 and α-syn, placing them in a common critical pathway of disease.

Tyrosine kinases

Tyrosine phosphorylation is an ubiquitous process that regulates cellular signaling and particularly cell growth. Tyrosine phosphorylation of α-syn has been less studied than its serine phosphorylation, and the functional consequences are less clear. Src and Fyn phosphorylate at Tyr125 (Ellis et al., 2001; Nakamura et al., 2001), and the Src family kinases Lyn and Frg have also been shown to phosphorylate it at Tyr125 (Negro et al., 2002). Furthermore, Syk has been demonstrated to more efficiently phosphorylate at residues Tyr125, Tyr133, and Tyr136 (Negro et al., 2002).

Regulation of α-syn phosphorylation – phosphatases

Protein phosphorylations are reversed by phosphatases. Despite the interest in the phosphorylation state of α-syn, there have been relatively few studies to determine the specific phosphatases that dephosphorylate α-syn. Thus far, protein phosphatase 2A (PP2A) has most convincingly been demonstrated to dephosphorylate p-Ser129 α-syn (Lee et al., 2011b), although other reports have suggested that it is not active on the protein (Waxman and Giasson, 2008; Lou et al., 2010). One study reported PP2C to be active at dephopshorylating α-syn at this site (Waxman and Giasson, 2008). The differences in studies may relate to different phosphatase compositions and the systems used to study effects.

Phosphoprotein phosphatase 2A (PP2A)

PP2A is an ubiquitous serine/threonine phosphatase with a broad range of substrates and functional roles. Specificity is achieved by variations in associated regulatory B subunits, with four gene families and multiple splice variants giving rise to differential pools that target different substrates. We found that a PP2A isoform that contains the Bα (PR55) subunit has strong ability to dephosphorylate α-syn at Ser129, while isoforms with other B subunits are inactive (Lee et al., 2011b). Assembly of Bα-containing PP2A is regulated by reversible methylation at the C-terminal leucine residue of PP2A's catalytic C subunit, providing a mechanism to control levels of the PP2A isoform that dephosphorylates α-syn (Tolstykh et al., 2000; Wu et al., 2000; Lee et al., 2011b). PP2A is a major serine/threonine phosphatase in the brain and is the major phosphatase that acts toward tau (Liu et al., 2005). As with α-syn, Bα-containing PP2A is critical for tau dephosphorylation (Xu et al., 2008) and, therefore, this isoform appears important in both Alzheimer's and Parkinson's diseases.

Other phosphatases

PP2C is another widely expressed phosphatase with multiple functional roles (Lammers and Lavi, 2007). It has been demonstrated to dephosphorylate α-syn at Ser129, but only of soluble, not fibrillar forms (Waxman and Giasson, 2008). The study of other phosphatases has been limited, although PP1 and PP2B do not appear to dephosphorylate α-syn (Waxman and Giasson, 2008; Lee et al., 2011b); the possibility that PP4 and PP5 could play a role cannot be discounted. No studies have reported the tyrosine phosphatases that act upon α-syn.

Functional consequences of α-syn phosphorylation

Hyperphosphorylation of key proteins appears to be a common event in multiple neurodegenerative disorders. Therefore, understanding the consequences of α-syn phosphorylation can help elucidate the pathogenesis of PD and a range of related conditions. We still do not fully know the physiological functions of α-syn, which will be important in ultimately understanding its pathophysiological roles. Unlike tau, whose phosphorylation at certain residues is critical for its normal functions in binding to microtubules and regulating axonal transport (Avila et al., 2004), it is unclear whether α-syn phosphorylation plays a physiological purpose. However, studies have investigated how phosphorylation can affect α-syn aggregation and toxic properties, both of which appear to be important disease determinants.

α-Syn aggregation

α-Syn is thought of as an intrinsically unfolded protein, but recent studies have demonstrated that it may occur natively as an α-helical tetramer (Bartels et al., 2011; Wang et al., 2011). Such tetrameric forms are resistant to aggregation (Bartels et al., 2011). Whether phosphorylation is involved in tetramer formation has not been established. Nonetheless, phosphorylation does play a significant role in α-syn aggregation as indicated by a number of studies *in vitro* and *in vivo*. Caution must be exercised in interpreting some studies due to the finding that point mutations to mimic or abolish phosphorylation do not accurately reflect native phosphorylation status (Paleologou et al., 2008).

Studies *in vitro* have led to variable conclusions as to the role of phosphorylation. One study demonstrated that phosphorylation at Ser129 can promote α-syn fibrillization (Fujiwara et al., 2002). However, targeted studies indicate that substitution of Ser129Asp or Ser129Glu does not affect fibrillization, while phosphorylation at this site inhibits fibrillization (Paleologou et al., 2008). Contrastingly, the Ser87Asp phosphomimic behaves similarly to native phosphorylation at this site, inhibiting fibrillization (Paleologou et al., 2008). These studies implicate phosphorylation in modulating α-syn aggregation, but are indicative of the complexities and potential interplay between different phosphorylation sites.

Similar to *in vitro* findings, a number of *in vivo* studies suggest differing consequences of phosphorylation. The majority of these studies have used point mutations as the most straightforward means of investigation, but these are complemented with studies that utilize the overexpression of α-syn kinases or pharmacological tools to modulate the phosphorylation of native proteins. In the human brain, phosphorylation at Ser129 is evident in α-synucleinopathies (Fujiwara et al., 2002; Anderson et al., 2006). In cultured cells, phosphorylation of α-syn appears to enhance its aggregation in combination with synphilin-1, with a Ser129Ala mutant form being unable to aggregate (Smith et al., 2005). In *Drosophila*, the phosphomimetic Ser129Asp resulted in increased levels of α-syn oligomers, but no increase in fibrillization compared to wild-type protein (Chen and Feany, 2005). However, in a rat model where the same mutation is overexpressed, there have been reports of no effects on aggregation (Azeredo da Silveira et al., 2009; McFarland et al., 2009). The relevance of these effects must be considered against the fact that point mutations may not faithfully recreate the effect of phosphorylation. To extend these results beyond mutations, there are interesting findings with overexpression of relevant kinases. For example, overexpression of GRK2 in *Drosophila* led to increased Ser129 phosphorylation and α-syn aggregation (Chen and Feany, 2005). Investigation of PLK2, a major α-syn kinase, has also been studied *in vivo*, demonstrating relevance of this kinase in α-syn phosphorylation, without report of effects on its oligomerization or aggregation (Inglis et al., 2009). In cells, no dramatic effect on aggregation has been observed by modulating phosphorylation with overexpression of PLKs or their inhibition with compounds such as BI2536 (Waxman and Giasson, 2011). On the other hand, we have demonstrated that, in α-syn transgenic mice, modulating the phosphatase relevant for reducing α-syn phosphorylation at Ser129 decreased phosphorylation, and this correlated with diminished formation of high-molecular-weight aggregated α-syn species (Lee et al., 2011b). Together, these studies still leave unanswered questions as to the role of phosphorylation on aggregation, with its consequences appearing to differ depending on species and means of manipulation. Some of these differences may result from the focus on just Ser129. It is likely that the interplay between phosphorylation at this site and others may influence overall aggregation state. Indeed, in the *Drosophila* model, preventing Tyr125 phosphorylation with a Tyr125Phe substitution increased α-syn aggregation, while mimicking phosphorylation at Ser129 with Ser129Asp substitution reduced its aggregation (Chen et al., 2009).

α-Syn toxicity

Whether aggregation of a pathogenic protein is intimately linked to toxicity has been another question that has plagued the neurodegeneration field. The current prevailing hypothesis is that small oligomers are toxic, whereas larger fibrils and insoluble aggregates may act as sinks to prevent toxicity (Haass and Selkoe, 2007). Numerous studies have looked at the roles of phosphorylation in α-syn toxicity, which could be dependent on, or independent of, its aggregation status. Studies in *Drosophila* convincingly demonstrated that Ser129 phosphorylation, either by GRK2 or a phosphomimetic mutation, induced neuronal toxicity (Chen and Feany, 2005). Furthermore, in studies in which Ser129 phosphorylation is reduced by enhancing PP2A activity, there is neuroprotection and behavioral improvement in α-syn transgenic mice (Lee et al., 2011b). In contrast, with viral vector-mediated overexpression of Ser129Asp, there was no toxicity observed in rats, while the un-phosphorylated mimic Ser129Ala mutation resulted in increased neurodegeneration in two studies (Gorbatyuk et al., 2008; Azeredo da Silveira et al., 2009), but neither construct had an effect in another study (McFarland et al., 2009). As with aggregation, such discrepancies observed in neurodegeneration may relate to species differences, other modifications to α-syn, and different experimental paradigms. Nonetheless, in PD, DLB, and MSA affected brains, it is not disputed that hyperphosphorylated α-syn is associated with the neurodegenerative pathology.

α-Syn localization

α-Syn is believed to be localized to membranes where it binds to phospholipids (Davidson et al., 1998), leading to its potential roles in vesicle trafficking and synaptic dysfunction. Studies have recently investigated the role of phosphorylation in modulating its membrane-related properties. Phosphorylation at Ser129, either through mutations or modulation of native phosphorylation state, appeared to have no effect on its ability to bind to membranes (Visanji et al., 2011). However, Ser129 phosphorylation of α-syn proteins that harbor additional mutations linked to PD pathogenesis (Ala53Thr or Ala30Pro) increased membrane association

(Visanji et al., 2011). This further indicates the importance of the interplay among residues within α-syn. Increased membrane localization may influence vesicle trafficking properties. For example, α-syn has been postulated to form pores in membranes that can lead to leakage of dopamine from vesicles into the cytosol causing toxicity (Lashuel et al., 2002; Volles and Lansbury, 2002).

α-Syn interactions with other proteins

Phosphorylation can affect the partners that α-syn is able to interact with. A peptide pulldown/mass spectrometry-based proteomic study demonstrated that Ser129 and Tyr125 phosphorylated α-syn preferentially interact with cytoskeletal proteins such as actin and vesicular trafficking proteins such as the clathrin heavy chain and AP2, while unphosphorylated α-syn interacts with mitochondrial electron transport proteins (McFarland et al., 2009). Although the significance of these interactions has not been confirmed, these results clearly illustrate the potential for phosphorylation to affect important protein-protein interactions critical to α-syn function. Other known interactions of α-syn do not appear to be regulated by its phosphorylation, such as that with synphilin-1 (Lee et al., 2004).

Therapeutic targeting of α-syn phosphorylation

As α-syn is intimately linked to PD pathogenesis, it is a clear target for therapeutic intervention. Phosphorylation provides an obvious drugable mechanism with numerous examples of pharmaceutical precedence (Cohen, 2002; McConnell and Wadzinski, 2009). As we have discussed in this review, it is evident that the phosphorylation of α-syn has complex consequences that remain to be fully understood. However, it is also clear that hyperphosphorylation, particularly at Ser129, correlates with disease progression, is present in the pathological hallmark lesions of α-synucleinopathies, and can have undesirable functional consequences. To reduce this Ser129 phosphorylation, inhibiting the relevant kinases has been a primary focus. In mice, treatment with the specific PLK inhibitor BI2356 inhibited α-syn phosphorylation at Ser129 (Inglis et al., 2009). However, there have been no further reports of the functional consequences of this inhibition or long-term safety of this approach. As the kinases that phosphorylate α-syn have ubiquitous distribution and there is evident redundancy with multiple kinases phosphorylating at the same site, it may be difficult to generate a safe and efficacious agent that has activity in the brain. Kinases are a critical class of targets for central nervous system disorders; however, issues of selectivity and blood-brain barrier permeation will prove major challenges to future development (Chico et al., 2009). Using allosteric activators to increase Ser129 phosphatase activity is another viable approach. This has been achieved *in vivo* by treatment with eicosanoyl-5-hydroxytryptamide (EHT) which inhibits demethylation of PP2A, thereby enhancing its phosphatase activity toward α-syn (Lee et al., 2011b). In α-syn transgenic mice on a diet containing EHT, Ser129 phosphorylation was reduced and α-syn aggregation was inhibited with concurrent improved neuronal integrity, reduction in inflammation, and amelioration of behavioral deficits (Lee et al., 2011b). This mechanism appears to be safe and selective as it only modulates a pool of PP2A, regulating a defined set of substrates. Importantly, PP2A is highly methylated in healthy cells, and methylation is only deficient in states of cellular stress associated with disease or injury.

Conclusions

Phosphorylation appears to play a major role in the pathological abnormalities of α-syn that underlie PD and related α-synucleinopathies. As we further understand the functional consequences of phosphorylation, the interplay between different phosphorylation sites, and the pathways that modulate phosphorylation, there is great potential to develop novel and efficacious therapeutics for these disabling diseases.

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