Biochemical and Biophysical Research Communications 418 (2012) 156–160

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Superiority of PLK-2 as α-synuclein phosphorylating agent relies on unique specificity determinants

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ARTICLE INFO

Article history:
Received 29 December 2011
Available online 10 January 2012

Keywords:
α-Synuclein
PLK2
PLK3
CK2
Parkinson's disease

ABSTRACT

Phosphorylation of α-synuclein at Ser-129 is of crucial relevance to Parkinson's disease and related synucleinopathies. Here we provide biochemical evidence that PLK2 and to a lesser extent PLK3 are superior over CK2, as catalysts of Ser-129 phosphorylation both in full length α-synuclein and in a peptide reproducing the C-terminal segment of the protein. By using substituted peptides we also show that the sequence surrounding Ser-129 is optimally shaped for undergoing phosphorylation by PLK2, with special reference to the two acidic residues at positions n−3 (Glu-126) and n+2 (Glu-131) whose replacement with alanine abrogates phosphorylation.

1. Introduction

Phosphorylation of α-synuclein at Ser-129, close to its C-terminal end is held as a hallmark of Parkinson's disease and related synucleinopathies mainly because α-synuclein within Lewy bodies, the inclusions that characterize these diseases, is extensively phosphorylated at this residue [1–5]. Indeed phospho-Ser-129 is the most extensive and consistent modification found in a survey of synuclein in Lewy Bodies [6]. Several protein kinases have been proposed to be responsible for α-synuclein phosphorylation at Ser-129, including G protein coupled receptor kinases [7], protein kinase CK1 [2] and CK2 [2,8,9]. CK2 looked particularly attractive also because the sequence surrounding α-synuclein Ser-129 includes several acidic residues, a typical feature of CK2 sites. Intriguingly however the most crucial acidic determinant for CK2 recognition, the one at position n+3 [10,11] is replaced by a non acidic residue (Gly-132) in the Ser-129 site, and, accordingly, α-synuclein phosphorylation by CK2 was found to be a relatively slow event [12]. It was also suggested that such a modest phosphorylation efficiency by CK2 could be enhanced by previous phosphorylation of tyrosyl residue(s) near-by, with special reference to Tyr-133 at position n+4 relative to Ser-129 [12].

More recently however robust evidence has been provided that the kinase(s) responsible for α-synuclein phosphorylation in live cells are members of the polo like kinase (PLK) family, with special reference to PLK2 and PLK3 [13,14]. While the experimental arguments supporting the *in vivo* implication of PLK2 and/or PLK3 are quite strong, the consensus sequences extracted from a peptide library, where acidic residues are selected at nearly all positions by either PLK2 or PLK3 [15] do not entirely conform to the sequence found around α-synuclein Ser-129. These observations prompted us to undertake an *in vitro* study aimed at disclosing structural features affecting the phospho-regulation of α-synuclein Ser-129 by either CK2 or PLK2 and PLK3.

The results, described in this report, show that PLK2 and to a lesser extent PLK3 are superior to CK2, as catalysts of α-synuclein phosphorylation, and that the sequence surrounding Ser-129 is optimally shaped for being phosphorylated by PLK2 and PLK3 but not by CK2.

2. Materials and methods

2.1. c-DNA constructs

Full-length PLK2 cDNA inserted into pX-HA [16] is a gift from Dr. Ingrid Hoffmann (German Cancer Research Center, Heidelberg, Germany). Full length PLK3 inserted pCEFL-HA vector [17] is a gift from Dr. Pedro A. Lazo (Instituto de Biología Molecular y Celular del Cáncer, CSIC-Universidad de Salamanca, Spain). pcDNA3.1-CK2α-myc-his was described previously [18].

2.2. Expression and purification of recombinant kinases

For the preparation of the GST-PLK2, GST-PLK3, plasmids, the DNA encoding full-length kinase was amplified from pX-HA-PLK2 and pCEFL-HA-PLK3 respectively by PCR where Bamh1 and XhoI

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doi:10.1016/j.bbrc.2011.12.152

restriction sites have been added to PCR primer and inserted into the pGEX4T1 vector at Bamh1 and XhoI sites.

For the preparation of the GST-CK2α plasmid the DNA encoding full-length kinase was amplified from pcDNA3.1-CK2α-myc-his by PCR where EcorI and NotI restriction sites have been added to PCR primer and inserted into the pGEX4T1 vector at EcorI and NotI sites.

All GST epitope tagged fusion proteins were expressed in BL-21 cells. Cells were growth at 37 °C to A600 nm = 0.6–0.8, and protein expression was induced with 0.5 mM Isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 5 h before harvest. Cell were lysed in 50 mM Tris–HCl (pH 8), 1 mM EDTA, 150 mM NaCl, 1 mM DTT and 1% Triton X-100, and by sonication (3 times for 10 s pulse in an ice bath with a Fisher Sonic Dismembrator 300 set at 35% maximal energy). After 30 min at 4 °C the lysate was centrifuged at 15000 g for 15 min at 4 °C. The supernatant was collected and incubated with glutathione Sepharose 4B resin (GE Healthcare) for 4 h at 4 °C and extensively washed with PBS. GST-fusion protein was eluted in 75 mM Hepes (pH 8) and 10 mM GSH. Recombinant alpha synuclein was described in [12].

2.3. In vitro phosphorylation

Reaction conditions for peptide phosphorylation experiments were the following: a range of concentration of synthetic peptides derived from human α-synuclein region (124–140) were phosphorylated by incubation in a 30-μl volume containing 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, and 50 μM [$\gamma-^{33}$P]ATP (specific radioactivity ~2000 cpm/pmol). The reaction was started by the addition of protein kinases (100 ng). The reaction mixtures were incubated for 10 min at 30 °C and stopped by ice cooling and absorption on phospho-cellulose p81 paper. Papers were washed three times with 75 mM phosphoric acid, dried, and counted in a scintillation counter.

Full-length α-synuclein phosphorylation were assayed in the same buffer above described. The reaction was started by the addition of protein kinases (100 ng PLK2 or 25 ng PLK3, or 10 ng CK2α or ng CK2α 10 plus 20 ng CK2β), incubated for 10 min at 30 °C and stopped by the addition of Laemmli buffer and boiling. Samples were analyzed by SDS–polyacrylamide gel electrophoresis and Coomassie staining. Gels were dried and exposed overnight to a multipurpose storage phosphor screen and analyzed using a Cyclone storage phosphor system (Packard). ^{33}P incorporation was evaluated by excision of protein bands from the gel followed by counting in a scintillation counter.

Initial rate data were fitted to the Michaelis–Menten equation with the program Prism (GraphPad Software, La Jolla, CA) to obtain K_m and V_{max} values. All results are reported as mean values ± SD of three determinations. Student's *t*-test was conducted to determine statistical significance.

2.4. Peptide synthesis

The solid-phase synthesis of the 33 amino acids C-terminal fragment of human synuclein (syn-33) and its derivatives including the phospho-peptide syn-17p133 was carried out on a fully automatic parallel peptide synthesizer (Model Syro II, Multi-SynThech GmbH, Witten, Germany) employing Fmoc chemistry [19] with 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as coupling reagent. The side-chain protected amino acid building blocks used were: Fmoc-Glu(*tert*-butyl), Fmoc-Asp(*tert*-butyl), Fmoc-Ser(*tert*-butyl), Fmoc-Tyr(*tert*-butyl), Fmoc-Tyr(PO(OBzl)OH), Fmoc-Gln(trityl), Fmoc-Asn(trityl) and Fmoc-Arg(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). Cleavage of the peptides was performed by reacting the peptidyl-resins with a mixture containing TFA/H_2O/thioanisole/ethanedithiol/phenol (10 ml/0.5 ml/0.5 ml/0.25 ml/750 m g) for 2.5 h. Crude peptides were subsequently purified by preparative HPLC (RP-18) with water/acetonitrile gradients containing 0.1% TFA and characterized by analytical HPLC and MALDI-MS. The purity of the peptides was in the range 95–98%.

3. Results

Approximately equi-active amounts of CK2, PLK2 and PLK3, as judged from similar activity toward the common substrate casein, were tested for their ability to phosphorylate α-synuclein. The results are on display in Fig. 1A where the phospho-radiolabeled bands of casein and α-synuclein generated by the three kinases are shown. Phosphorylation of α-synuclein normalized toward that of casein is shown in Fig. 1B. The data unambiguously show that PLK2 is an α-synuclein phosphorylating agent superior to PLK3 and, even more, to CK2 whose aptitude to phosphorylate α-synuclein is almost negligible as compared to its casein kinase activity.

To show that α-synuclein phosphorylation mainly if not entirely takes place at Ser-129, advantage has been taken of a mutant in which Ser-129 is replaced by a non phosphorylatable residue (α-syn S129A). This mutant is entirely unaffected by both PLK2 and PLK3 while its phosphorylation by CK2 is less than 10% as compared to wild type (not shown).

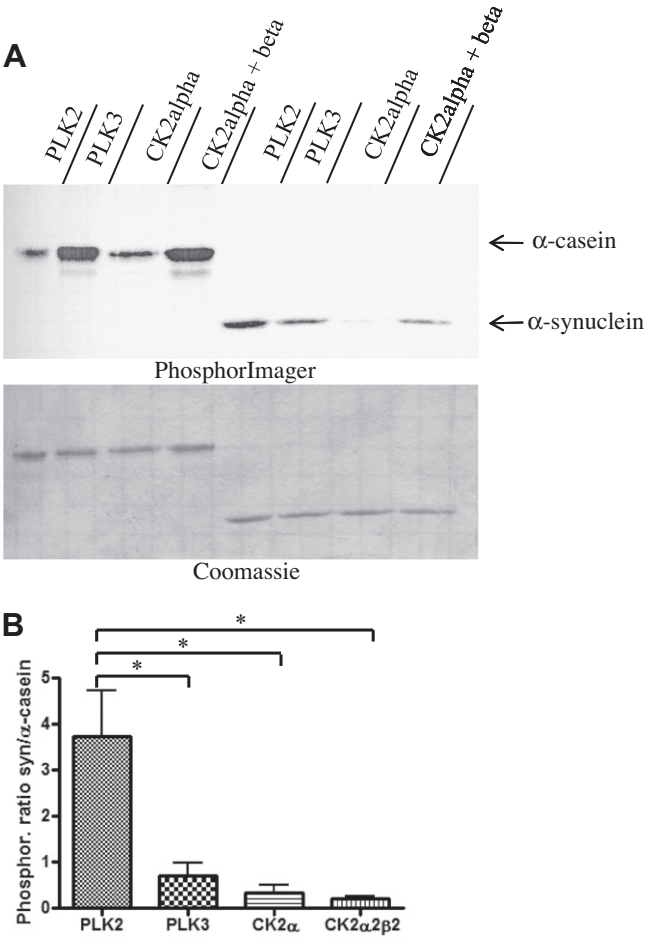

Fig. 1. Phosphorylation of α-casein and α-synuclein by PLK2, PLK3 and CK2. (A) 1.5 μg of α-casein or 0.5 μg of α-synuclein were incubated in the absence or presence of recombinant PLK2 (100 ng), PLK3, (25 ng), CK2α (10 ng) or CK2α (10 ng) plus CK2β (20 ng) in a radioactive mixture as described in the Section 2. Proteins were resolved by SDS/PAGE, coomassie stained, and analyzed by PhosphorImager. (B) Phosphorylation of α-synuclein normalized toward that of casein is shown (mean values ± SD of three determinations, $^*p < 0.05$).

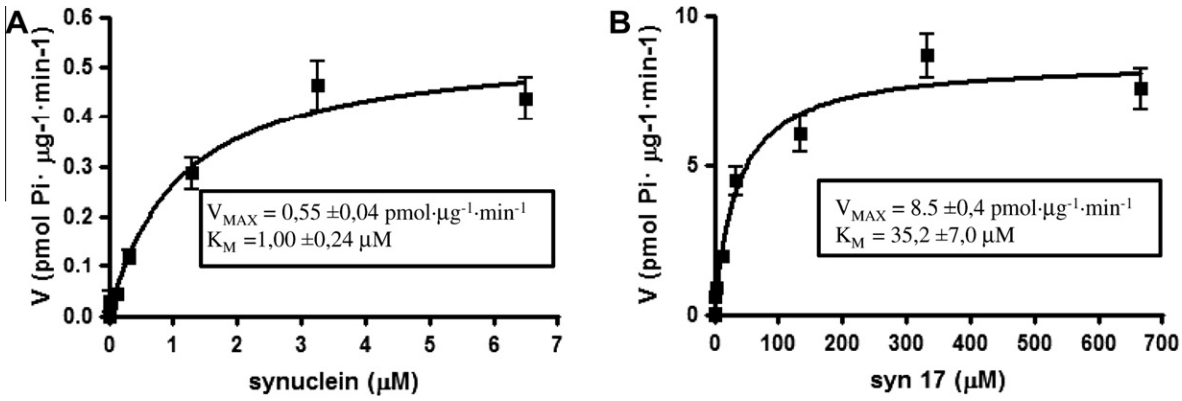

Fig. 2. Phosphorylation kinetics of α-synuclein and its c-terminal peptide by PLK2. (A) Increasing concentrations of α-synuclein were phosphorylated by PLK2 in the radioactive mixture for 10 min at 30 °C. Samples were loaded on SDS/PAGE, coomassie stained and analyzed by PhosphorImager. Radioactive bands were cut and radioactivity incorporation was analyzed in a scintillation counter (mean values ± SD of three determinations). (B) Increasing concentrations of Syn-17 peptide (encompassing the C-terminal segment of α-synuclein, from residue 124 to the C-terminus) was phosphorylated by PLK2 in the radioactive mixture for 10 min at 30 °C and the radioactivity incorporated into the peptide was determined as described in Section 2.

The good performance of PLK2 as an α-synuclein kinase is corroborated by the kinetics shown in Fig. 2A where α-synuclein displays a K_m = 1.0 μM, a value compatible with its phosphorylation by PLK2 under physiological conditions, its concentration in normal human brain being 1 μM [20].

In order to gain information about the structural features determining the targeting of α-synuclein by PLK2 a 17-mer peptide encompassing the C-terminal segment of α-synuclein, inclusive of Ser-129, was synthesized and assayed as a substrate of PLK2. As shown in Fig. 2B the peptide is readily phosphorylated with a V_{max} one order of magnitude higher than that of full length α-synuclein itself. The K_m of the peptide however (35.2 μM) is more than 30-fold higher than that of the full size protein, suggesting that remote docking site(s) play also a role in α-synuclein recognition by PLK2. On the other hand the peptide is almost unaffected by CK2, to such an extent that a reliable kinetic analysis turned out to be impossible.

To investigate the local structural features responsible for its phosphorylation by PLK2 the α-synuclein peptide was subjected to substitutions of acidic residues suspected to play a role as specificity determinants based on previous studies with peptide libraries [15]. In addition a derivative in which the Gly at position n+3 was replaced by Glu, thus generating the CK2 consensus (S/T-x-x-E/D) and one in which Tyr133 (at n+4) position, shown to be an *in vitro* target for the tyrosine kinase Syk [12], was replaced by phospho-tyrosine, were also generated.

As shown in Table 1 the replacement of Glu at position n-3 (relative to Ser-129) and, to a lesser extent of Glu at position n+2 is detrimental, the former in particular giving rise to a peptide which has lost any aptitude to be phosphorylated by PLK2 (compare peptides 2 and 4 with peptide 1). By contrast glutamic acid at n+1 can be replaced by Ala without any appreciable consequence (compare peptides 1 and 3) and the replacement of Gly at position n+3 with glutamic significantly worsens peptide phosphorylation by PLK2, while evoking phosphorylation by CK2, as expected (compare peptides 5 and 1). Another substitution rendering the parent (wild type) peptide susceptible to detectable phosphorylation by CK2 is that of the EE doublet adjacent to the C terminus of Ser-129 with two aspartic acids (peptide 6), consistent with the observation that in atypical CK2 phosphosites lacking the acidic determinant at n+3 Asp tends to prevail over Glu at positions n+1/+2 [21].

Conversely the replacement of Tyr-133 with phosphotyrosine (peptide 7) is unable by itself to evoke CK2 dependent phosphorylation, while significantly increasing the phosphorylation of the parent peptide by PLK2, albeit not by PLK3, whose responsiveness to substitutions is otherwise quite similar to that of PLK2.

A kinetic analysis performed with the substituted peptides (Table 2) reveals that the observed alterations in phosphorylation by PLK2 are mostly accounted for by drops in V_{max}, particularly evident upon substitution of glutamyl residues at positions n−3 and

Table 1
Effect of altering the sequence of the α-synuclein c-terminal peptide on its phosphorylation rate by PLK2, PLK3, and CK2α. Phosphorylation rate is expressed as percentage of that of the parent wild-type peptide by PLK2. All peptides were tested at 333 μM concentrations under conditions detailed in Section 2. Data presented are the mean of triplicate assays. The target residue (syn Ser-129) is bold typed; substitutions relative to the parent peptide are underlined.

n	Peptide	PLK2	PLK3	CK2α
1	RRRAYEMP**S**EEGYQDYEPEA	100	26.5	4.4
2	RRRAY<u>A</u>MP**S**EEGYQDYEPEA	1.3	0.9	4.3
3	RRRAYEMP**S**<u>A</u>EGYQDYEPEA	91	26.3	N.D.
4	RRRAYEMP**S**E<u>A</u>GYQDYEPEA	25.6	4.8	2.3
5	RRRAYEMP**S**EE<u>E</u>YQDYEPEA	66.7	31.3	104.1
6	RRRAYEMP**S**<u>DD</u>GYQDYEPEA	90.7	17.4	22.7
7	RRRAYEMP**S**EEG<u>pY</u>QDYEPEA	144	24.4	4.6

Table 2
Kinetic constants for the phosphorylation of α-synuclein c-terminal peptides derivatives by PLK2, PLK3 and CK2α. K_m and V_{max} were calculated from three independent experiments and expressed in μM and in pmol Pi·μg^{-1}·min^{-1}, respectively.

n	Peptide		PLK2	PLK3	CK2
1	RRRAYEMPSEEGYQDYEPEA	V_{max}	8.25 ± 0.4	2.4 ± 0.1	N.D.
		K_m	35.2 ± 7.0	66.2 ± 14	N.D.
2	RRRAY<u>A</u>MPSEEGYQDYEPEA	V_{max}	N.D.	N.D.	N.D.
		K_m	N.D.	N.D.	N.D.
3	RRRAYEMPS<u>A</u>EGYQDYEPEA	V_{max}	7.9 ± 0.3	2.5 ± 0.2	N.D.
		K_m	39 ± 5.6	72 ± 18.5	N.D.
4	RRRAYEMPSE<u>A</u>GYQDYEPEA	V_{max}	2.2 ± 0.1	N.D.	N.D.
		K_m	11.7 ± 1.9	N.D.	N.D.
5	RRRAYEMPSEE<u>E</u>YQDYEPEA	V_{max}	5.6 ± 0.2	3.2 ± 0.2	38.93 ± 5.9
		K_m	13.8 ± 3.9	44 ± 10.1	1198 ± 292
6	RRRAYEMPS<u>DD</u>GYQDYEPEA	V_{max}	8.6 ± 0.2	1.5 ± 0.1	12.2 ± 1.8
		K_m	38.4 ± 3.8	20.9 ± 5	1807 ± 374
7	RRRAYEMPSEEG<u>pY</u>QDYEPEA	V_{max}	16.9 ± 0.6	2.3 ± 0.1	N.D
		K_m	93.9 ± 12.3	70 ± 12.6	N.D.

n+2 with alanine. In the former case phosphorylation was flatly undetectable up to 0.65 mM peptide concentration (Supplementary material Fig. S1) hampering any kinetic determination. To note that reliable kinetics with CK2 could be performed only with the substituted peptides 5 and 6. With both however the K_m values were exceedingly high (>1 mM) denoting a very poor phosphorylation efficiency, despite the high V_{max} value. In contrast kinetic constants of the peptides with PLK3 roughly parallel those observed with PLK2, denoting in general a significantly lower phosphorylation efficiency.

In conclusion our analysis with substituted peptide substrates highlights the crucial importance of the two glutamic acid residues at positions n−3 and n+2 to ensure efficient phosphorylation of Ser-129 by PLK2. The same acidic residues are also critical for PLK3 mediated phosphorylation as outlined in Table 1 and 2. By sharp contrast they are not sufficient to ensure any substantial phosphorylation by CK2 which in order to appreciably phosphorylate Ser-129 needs the replacement of the naturally occurring Gly132 with a glutamic acid.

4. Discussion

The data presented provide unambiguous biochemical evidence that PLK2 and to a lesser extent PLK3 are superior to CK2 as far as their ability to phosphorylate Ser-129 of α-synuclein is concerned. Firstly in fact PLK2 phosphorylates full size α-synuclein much more readily than equi-active amounts of CK2, normalized against the common substrate casein. Secondly the K_m for α-synuclein phosphorylation by PLK2 (1 μM) is compatible with a physiological role of PLK2 as α-synuclein kinase. Thirdly the highly conserved sequence around α-synuclein Ser-129 looks optimally shaped for PLK2 catalyzed phosphorylation since substitution of critical residues nearby is detrimental to phosphorylation. In contrast appreciable phosphorylation by CK2 only occurs if the natural sequence around Ser-129 is altered, with special reference to the replacement of Gly-132 with Glu, which instead leaves phosphorylation by PLK2 and PLK3 almost unaffected.

Altogether our data disclose critical differences between the consensus sequence of PLK2 and that of CK2: the latter, as already established by previous studies [10,11], displays an almost absolute requirement for an acidic side chain at position n+3, which instead is just tolerated by PLK2. In contrast phosphorylation of α-synuclein Ser-129 by PLK2 is critically relying on the two acidic side chains of Glu-126 and Glu-131, at positions n−3 and n+2, respectively.

Our present results in conjunction with previous data obtained with peptide libraries [15], definitely assign PLK2 and PLK3 to the small category of "acidophilic" Ser/Thr protein kinases, whose site recognition is critically relying on acidic residues, and whose members are often termed "casein kinases" after their aptitude to phosphorylate casein *in vitro* [10]. These are the Golgi apparatus casein kinase (G-CK), responsible for the phosphorylation of casein in the lactating mammary gland and the two pleioteropic and ubiquitous CK1 ("casein kinase 1") and CK2 ("casein kinase-2"). While however the consensus sequences of G-CK, CK1 and CK2 have been unambiguously defined in the past and specific peptide substrates for each of these were developed (e.g. [22,23]) the precise consensus of PLK2 (and PLK3) is still a matter of conjecture. Interestingly neither the specific peptide substrate of G-CK, nor those of CK2 and CK1 are phosphorylated to any appreciable extent by amounts of PLK2 sufficient to readily phosphorylate the α-synuclein peptide (see Fig. S2). A possible explanation, consistent with our data presented in Tables 1 and 2 would be that the minimum consensus for PLK2 is E-x-x-S-x-E/D, i.e. strictly relying on both the acidic determinants at n−3 and n+2 which are not simultaneously present in any of the peptide substrates of the other "casein kinases". It should be also noted in this respect that the information drawn from our substituted peptide substrates is only partially overlapping the PLK2 consensus obtained from a peptide library approach [15]. This latter in fact includes acidic residues not only at positions n−3 and n+2, but also at additional positions, notably n+1 and n+3 where acidic residues do not improve Ser-129 phosphorylation (see Table 1). This way reflect the fact that while our information is drawn from an individual PLK2/PLK3 phosphorylation site, the consensus calculated by Johnson et al. [15] was drawn from a library of 720 peptides derived from human protein phosphorylation sites.

It should be finally mentioned that our data strongly support the view that α-synuclein recognition by PLK2 critically relies also on remote interactions, since the affinity of PLK2 for the full size protein is 30-fold higher than that for the 17 residues long peptide encompassing the target serine (K_m 1 vs > 35 μM). This is a quite common feature among protein kinases (see e.g. [24–26]), and in our case it does not entirely come as a surprise considering that the N-terminal region of α-synuclein (residues 1–95) has been shown to make specific interactions with PLKs [14]. In the perspective of understanding the role of PLKs mediated phosphorylation of α-synuclein in the context of the Parkinson's disease, it will be important to precisely map the structural elements responsible for such reciprocal interactions outside the phosphorylation site. This may also pave the road toward the development of highly selective inhibitors disrupting these interactions and therefore hampering α-synuclein Ser-129 phosphorylation without affecting the phosphorylation of other PLK2/PLK3 targets. Compounds like these may display therapeutic potential in neurodegenerative diseases.

Acknowledgments

We thank Dr. Ingrid Hoffmann for providing us the pX-HA-PLK2 vector and Dr. Pedro A. Lazo for providing us the pCEFL-HA-PLK3 vector.

This work was supported by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC) to L.A.P.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.152.

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