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Identification and functional characterization of Polo-like kinase 2 autoregulatory sites

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Abstract

Polo family kinases play important roles in cellular proliferation as well as neuronal synaptic plasticity. However, the posttranslational regulation of these kinases is not fully understood. Here, we identified several novel Plk2 phosphorylation sites stimulated by Plk2 itself. By site-directed mutagenesis, we uncovered three additional hyperactivating Plk2 mutations as well as a series of residues regulating Plk2 steady-state expression level. Because of the established role of Plk2 in homeostatic negative control of excitatory synaptic strength, these phosphorylation sites could play an important role in the rapid activation, expansion, and prolongation of Plk2 signaling in this process.

Keywords

Plk2; SNK; homeostatic plasticity; polo kinase; posttranslational modification

The polo family of serine/threonine kinases serves important roles in diverse processes such as cell proliferation, division and neuronal differentiation (Archambault and Glover, 2009, Draghetti et al., 2009) as well as synaptic signaling and plasticity in post-mitotic neurons (Seeburg et al., 2005, Inglis et al., 2009, Mbefo et al., 2009). Polo-like kinases (Plks) are often recruited with precise spatial and temporal regulation. For example, during the cell cycle, Plk1 expression is upregulated at the G2/M transition by transcriptional activation (Uchiumi et al., 1997). Following exit from mitosis, Plk1 levels are quickly reduced via ubiquitin-dependent proteolysis (Charles et al., 1998, Fang et al., 1998, Shirayama et al., 1998). This induction and destruction cycle is reminiscent of the regulation of Plk2 (also called serum-inducible kinase) in neurons. Plk2 is an immediate-early gene (Simmons et al., 1992) whose transcriptional expression in brain is robustly stimulated by high levels of synaptic activity (Kauselmann et al., 1999). Upon induction, Plk2 acts to homeostatically downregulate excitatory synapses and dendritic spines in a negative feedback loop by targeting several postsynaptic proteins including SPAR, a protein that promotes spine growth (Pak et al., 2001, Pak and Sheng, 2003, Seeburg et al., 2008, Lee et al., 2011). Plk2 levels can also be rapidly increased by inhibition of the proteasome in the absence of synaptic stimulation (Pak and Sheng, 2003), suggesting that Plk2 is constitutively restrained by the ubiquitin-proteasome system and unleashed during periods of intense overactivity.

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Because indiscriminate synapse loss can lead to profound cognitive dysfunction, the activity of Plk2 must be tightly controlled.

Despite the importance of regulating Plk functions, little is known regarding the posttranslational mechanisms that affect their activity. Polo kinases share a general primary structure consisting of an N-terminal kinase domain and a conserved C-terminal motif termed the polo box domain (PBD). The PBD is a characteristic feature of this family comprised of one or two polo boxes, binding modules important for interactions with target substrates and for proper subcellular localization (Elia et al., 2003a, Elia et al., 2003b). Within the kinase domain, Plks can be universally activated by phosphorylation of the T-loop / activation loop that is thought to relieve inhibitory intramolecular interactions between the kinase domain and the PBD (Qian et al., 1999, Jang et al., 2002b). The polo-like kinase Plx1 from *Xenopus laevis* also shows retarded gel electrophoretic mobility specifically during M phase, suggestive of posttranslational modification (Kelm et al., 2002), and a variety of other Plk phosphorylation events have been reported (Golsteyn et al., 1995, van de Weerd et al., 2005, Maroto et al., 2008). However, aside from the T-loop activation, the functional role of Plk phosphorylation remains unclear.

Here, we have identified several novel autoregulatory sites within Plk2. By site-directed mutagenesis of each phosphosite, we have characterized the role of these sites in Plk2 protein expression and kinase activity, and identified a new group of hyperactive mutants that reveal insight into the intramolecular mechanisms of polo-like kinase regulation.

Experimental Procedures

DNA constructs

Myc epitope-tagged Plk2, hyperactivating Plk2 (T236E), kinase-dead Plk2 (K108M), and SPAR were expressed from pGW1-CMV. Plk2 mutations were generated by site-directed mutagenesis (QuikChange, Stratagene) using the following oligonucleotides (5' to 3'): T295A (F): CAT AAG AGA AGC AAG ATA TGC AAT GCC GTC TTC ATT ACT G, (R): CAG TAA TGA AGA CGG CAT TGC ATA TCT TGC TTC TCT TAT G; T295E (F): CAT AAG AGA AGC AAG ATA TGA AAT GCC GTC TTC ATT ACT G, (R): CAG TAA TGA AGA CGG CAT TTC ATA TCT TGC TTC TCT TAT G; S298A (F): GCA AGA TAT ACA ATG CCG GCT TCA TTA CTG GCC CCC GCC, S298A (R): GGC GGG GGC CAG TAA TGA AGC CGG CAT TGT ATA TCT TGC; S298E (F): GCA AGA TAT ACA ATG CCG GAA TCA TTA CTG GCC CCC GCC, S298E (R): GGC GGG GGC CAG TAA TGA TTC CGG CAT TGT ATA TCT TGC; S299A (F): GAT ATA CAA TGC CGT CTG CAT TAC TGG CCC CCG CCA AG, S299A (R): CTT GGC GGG GGC CAG TAA TGC AGA CGG CAT TGT ATA TC, S299E (F): GAT ATA CAA TGC CGT CTG AAT TAC TGG CCC CCG CCA AG, S299E (R): CTT GGC GGG GGC CAG TAA TTC AGA CGG CAT TGT ATA TC; S321A (F): CCC AGA GGA CCG CCC CGC TTT GGA TGA CAT CAT TCG G, S321A (R): CCG AAT GAT GTC ATC CAA AGC GGG GCG GTC CTC TGG G; S321E (F): CCC AGA GGA CCG CCC CGA ATT GGA TGA CAT CAT TCG G, S321E (R): CCG AAT GAT GTC ATC CAA TTC GGG GCG GTC CTC TGG G; S386A (F): GAC ACA CAC AAT AAA GTG GCT AAG GAA GAT GAA GAC ATC, S386A (R): GAT GTC TTC ATC TTC CTT AGC CAC TTT ATT GTG TGT GTC; S386E (F): GAC ACA CAC AAT AAA GTG GAA AAG GAA GAT GAA GAC ATC, S386E (R): GAT GTC TTC ATC TTC CTT TTC CAC TTT ATT GTG TGT GTC; T413A (F): CAA CCC AGC AAA CAC AGA GCA GAT GAG GAG CTC CAG CCT C, T413A (R): GAG GCT GGA GCT CCT CAT CTG CTC TGT GTT TGC TGG GTT G; T413E (F): CAA CCC AGC AAA CAC AGA GAA GAT GAG GAG CTC CAG CCT C, T413E (R): GAG GCT GGA GCT CCT CAT CTT CTC TGT GTT TGC TGG GTT G; S497A (F): CAA AGA GCA GTT GAG CAC GGC CTT TCA GTG GGT CAC CAA ATG, S497A

(R): CAT TTG GTG ACC CAC TGA AAG GCC GTG CTC AAC TGC TCT TTG; S497E
 (F): CAA AGA GCA GTT GAG CAC GGA ATT TCA GTG GGT CAC CAA ATG, S497E
 (R): CAT TTG GTG ACC CAC TGA AAT TCC GTG CTC AAC TGC TCT TTG; S588A
 (F): GAT GGT GGC GAT CTC CCT GCT GTT ACT GAC ATT CGA AGA C, S588A
 (R): GTC TTC GAA TGT CAG TAA CAG CAG GGA GAT CGC CAC CAT C; S588E
 (F): GAT GGT GGC GAT CTC CCT GAA GTT ACT GAC ATT CGA AGA C, S588E
 (R): GTC TTC GAA TGT CAG TAA CTT CAG GGA GAT CGC CAC CAT C; T590A
 (F): GGC GAT CTC CCT AGT GTT GCT GAC ATT CGA AGA CCT CGG, T590A (R):
 CCG AGG TCT TCG AAT GTC AGC AAC ACT AGG GAG ATC GCC; T590E (F): GGC
 GAT CTC CCT AGT GTT GAA GAC ATT CGA AGA CCT CGG, T590E (R): CCG AGG
 TCT TCG AAT GTC TTC AAC ACT AGG GAG ATC GCC. All constructs were
 sequenced in their entirety to verify the correct mutation and confirm that no other
 inadvertent mutations were introduced.

Mass spectrometric identification of phosphorylation sites

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed by the Taplin Mass Spectrometry Facility (Harvard Medical School) essentially as described (Holt et al., 2009). Following LC-MS/MS analysis protein database searching was performed using Sequest software.

Antibodies

The following antibodies were purchased from commercial sources or have been described: mouse anti-AFP antibody (MP Biomedicals, LLC), rabbit anti-Myc antibody (Cell Signaling) (Pak and Sheng, 2003), rabbit anti-SPAR antibody (Pak et al., 2001). Alexa Fluor 488 and 555 (Invitrogen) were used as secondary antibodies for all immunofluorescence.

Cell culture and Transfection

COS7 cells (ATCC) were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 0.1% gentamicin (Invitrogen). COS7 cells were transiently transfected with 1 μ g of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. After expression for 24 hrs, cells were harvested in 1 $\times$ SDS loading buffer (50mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue). For cycloheximide experiments, 216 μ M cycloheximide was added to COS7 cells 16 hours after transfection with the different Plk2 constructs and harvested as above at the indicated time-points. To determine protein half-life, data was plotted on a log scale against time and $\tau_{1/2}$ was calculated from linear equations derived for each construct as described (Touitou et al., 2001). Primary hippocampal neurons were prepared at E18 and maintained 19–24 days in vitro (DIV). Cells were plated at medium density (~150 cells/mm²) on coverslips coated with poly-D-lysine (Sigma) and laminin (2 μ g/mL, Roche). Cultures were grown in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM glutamine and 12.5 μ M glutamate. Neurons were transfected at DIV 16 using Lipofectamine 2000 (Invitrogen). For transfection with Lipofectamine 2000, 2 μ g of DNA in 25 μ l serum-free Neurobasal media (Invitrogen) was mixed with 2 μ l of Lipofectamine 2000 in 25 μ l serum-free Neurobasal media that was pre-incubated for 5 min at room temperature. The DNA and Lipofectamine mixture was incubated for 20–30 min at room temperature and added dropwise into the media. Transfected neurons were incubated for 24 hrs before fixing.

Immunoblotting

Samples were separated by SDS-PAGE, transferred to nitrocellulose and blocked with 5% nonfat dry milk in 1X TBS containing 0.1% Tween-20. Blots were incubated with primary antibodies overnight at 4°C and Horseradish peroxidase-conjugated secondary antibodies

(Roche) for 1 hr at room temperature. Blots were imaged through enhanced chemiluminescence system (Western Lightning (Perkin Elmer) or SuperSignal West Femto (Pierce)). All immunoblotting experiments were conducted at minimum in triplicate, and analyzed at a range of exposures to ensure band intensities were within the linear range for ECL and autoradiography.

Immunocytochemistry

For immunolabeling, neurons were fixed 24 hrs post-transfection in 1% PFA for 5 min and -20°C methanol for 10 min. Primary antibodies for immunostaining were diluted in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate pH 7.4, 450 mM NaCl) and incubated overnight at 4°C . Secondary antibodies were also diluted in GDB and incubated with cover slips for 2 hrs at room temperature.

Quantification and image analysis

Microscopy images were acquired using an Axiovert 200M epifluorescence inverted microscope (Zeiss) using consistent camera exposure levels, respectively, for each fluorescent marker in each experiment. For image analysis and quantification, measurements were made using MetaMorph software (Molecular Devices). Average intensity was calculated from integrated intensity and area for each selected area. For immunostaining, analysis of primary and secondary dendritic branches was used for quantification. For spine analysis, Z stacks of dendritic images were captured using an LSM510 laser scanning confocal microscope (Zeiss). Reconstructed images generated by compressing collected Z stacks were analyzed. Spine density analysis was performed using automated NeuronStudio software (CNIC, Mount Sinai) as described (Rodriguez et al., 2008).

Sequence analysis and alignment

For sequence alignment of polo-like kinases we used ClustalW (Larkin et al., 2007). The WT-Plk2 sequence was used as a template for the calpain cleavage site prediction on the CaMPDB website (<http://calpain.org/index.rb>).

Statistical analysis

All values were expressed as mean $\pm$ S.E.M., if otherwise not mentioned. Experiments were performed at least in triplicate and Student's t-test used for all pair-wise tests of significance, or one-way ANOVA with Newman-Keuls *post hoc* test for multiple group comparisons.

RESULTS

Active Plk2 is highly phosphorylated in mammalian cells

Previous studies showed that wild-type (WT) Plk2 migrated with conspicuously different mobility analyzed by SDS-PAGE compared to kinase-dead (KD) Plk2 (K108M mutation in the active site), suggestive of post-translational modification (Pak and Sheng, 2003). Plk2 has also been shown to autophosphorylate via *in vitro* kinase reactions reconstituted entirely with purified components (Pak and Sheng, 2003, Lee et al., 2011). These observations implicated Plk2 autophosphorylation in regulating kinase function as well as tertiary conformation.

To determine phosphorylation sites within Plk2 stimulated by the kinase itself, we used a heterologous cell-based assay system to obtain sufficient material for mass spectrometric analysis. We transfected COS7 cells with myc-epitope tagged versions of constitutively active (CA) (T236E mutation in the T-loop), WT or KD-Plk2. Consistent with previous findings, these constructs exhibited a "stair-step" pattern, with KD-Plk2 migrating at a lower

position and in a tighter, more distinct band than WT Plk2, which appeared as a diffuse doublet, while CA-Plk2 migrated as an apparent single band at a higher position (Fig. 1A, left). These findings suggested that WT-Plk2 consisted of a mixed population of under-phosphorylated (fast migrating, or compact) and highly phosphorylated (slow migrating, or open) species. Thus, we performed preparative immunoprecipitations of KD-Plk2 (K108M) or CA-Plk2 (T236E) using myc-agarose resin and excised bands from Coomassie-stained SDS-PAGE gels (Fig. 1A, right) for trypsin digestion followed by liquid chromatography-tandem mass spectrometry.

The above analysis identified 9 candidate phosphorylated amino acid residues within active Plk2 that were not observed in kinase-inactive Plk2 (Fig. 1B) and thus represented potential autoregulatory sites. Peptides containing phosphorylated residues were identified based on the additional mass of a phosphate group, as well as the site on the peptide that is phosphorylated, although in some cases it was not possible to make a confident assignment. In such cases, possible alternate locations are shown (e.g. Fig. 1B, sites 2 and 7). It is also possible that some sites were phosphorylated but undetected due to sensitivity limitations. Four of the identified sites clustered near the carboxy end of the kinase domain (T295, S298, S299, and S321), 3 resided in the linker region between the kinase domain and the PBD (S386, T413, and S497), and 2 sites lay between the two polo boxes of the PBD (S588 and T590) (Fig. 1C). We performed the same analysis for the highly related kinase Plk3, and surprisingly found only 2 sites that were in common with those of Plk2 (Fig. 1C). Alignment of several Plk family members (Fig. 2) showed that the residues identified here are all novel with the exception of T295, the homologous site of which has been reported to be autophosphorylated in Plx1 (Kelm et al., 2002).

Phosphorylation sites in Plk2 regulate protein stability

To test the function of these identified serine / threonine phosphoresidues, we performed site-directed mutagenesis to convert each into either the nonphosphorylatable amino acid alanine, or the phosphomimic glutamic acid. We first tested the expression level of each mutant protein, because KD-Plk2 mutation led to higher steady-state protein accumulation than WT-Plk2 at equal amounts of transfected DNA (Fig. 1A). We transfected COS7 cells with each of the alanine or glutamic acid substitution mutants as well as KD-, CA- and WT-Plk2 controls and analyzed their expression level and gel migration. As before, KD migrated as a single band at the lowest position, WT as a doublet in the middle position, and CA as a single band at the highest position (Fig. 3A, lanes 11–13 and 14–16). Nearly all of the new Plk2 mutants migrated as doublets resembling WT-Plk2; the one exception was the S588E mutation which migrated as a single band at the same level as CA-Plk2 (Fig. 3A, compare lanes 22 and 16).

Quantification of total Plk2 levels expressed in COS7 cells revealed a complex pattern of individual mutants on the steady-state level of Plk2 (Fig. 3B). As expected, the kinase dead mutation, K108M, resulted in greater levels of Plk2. Surprisingly, expression of constitutively active Plk2 (T236E) also resulted in increased stability relative to WT-Plk2. Interestingly, the 3 non-phosphorylatable mutations very near to or within the PBD (S497A, S588A, and T590A; Fig. 3B lanes 19, 21, and 23) resulted in elevated levels of Plk2, while the corresponding phosphomimetic mutation for the same site had no discernible effect on protein level (Fig. 3B, lanes 20, 22, and 24). Conversely, S299A resulted in a significant decrease in stability of Plk2 expression, while the corresponding phosphomimetic mutation, S299E, resulted in increased stability relative to WT-Plk2 (Fig. 3B, lanes 5–6). The regulation at these 4 phosphosites suggests a bidirectional control of protein stability in COS7 cells.

Unexpectedly, many of the mutations in the kinase domain (T295, S298, S321) and linker region (S386, T413) resulted in greatly increased stability of Plk2 in both the phosphomimetic and non-phosphorylatable forms. While all of these mutations resulted in elevated expression of Plk2 even relative to KD and CA, the higher levels of these mutants may be exaggerated by the smearing together of signals from doublet bands. Thus, this analysis identified novel phosphosites that resulted in either significant alterations in protein stability with no effect on conformation, or markedly affected Plk2 spatial conformation but not expression level (S588E).

To further demonstrate changes in Plk2 stability, we transfected wild-type and selected mutants in COS7 cells for 16 hours followed by treatment with the protein synthesis inhibitor cycloheximide for different periods of time to monitor rates of Plk2 degradation by western blotting. These data show that mutants with increased steady-state levels of Plk2 relative to WT (KD, CA, S299E, and S588A) had increased half-lives, whereas mutants with slightly decreased or similar steady-state levels (S299A and S588E) had comparable half-lives to WT (Fig. 3C–D). We were not able to address the role of proteasomal degradation in Plk2 stability for any of the mutants because blockade of the proteasome causes increases in endogenous Plk2 expression to the same level as overexpressed Plk2 (data not shown). This confound prevents us from interpreting the results of such experiments in a meaningful way as this level of expression of endogenous WT Plk2 with proteasomal blockade will heavily compete with the transfected mutants.

Plk2 phosphorylation site mutants regulate kinase activity

To determine whether Plk2 kinase activity was affected by any of the mutations, we performed co-transfections with the known Plk2 direct phosphorylation substrate SPAR (Pak and Sheng, 2003). Because SPAR is phosphorylated and degraded by Plk2 via a proteasome-ubiquitin mechanism, extent of SPAR post-translational modification and reduction in expression can be used as an index of Plk2 phosphorylation. As expected, SPAR expression level was highest when co-expressed with KD-Plk2 (Fig. 4A, lanes 11 and 14; Fig. 4C). The SPAR band was reduced in intensity and gel shifted higher when co-expressed with WT-Plk2 (Fig. 4A, lanes 12 and 15; Fig. 4C), and was dramatically decreased in intensity and presented as a faint, high-migrating smear when co-expressed with CA-Plk2 (Fig. 4A, lanes 13 and 16; Fig. 4C). The new phosphosite mutants generally had modest effect on Plk2 activity against SPAR (Fig. 4A). Two notable exceptions occurred with mutant pairs S299A/S299E and S588A/S588E (Fig. 4A). With S299E, SPAR levels were significantly decreased, similar to that observed with CA-Plk2 (Fig. 4B and C). Interestingly, both the non-phosphorylatable and phosphomimetic mutants S588A and S588E caused a similar increase in Plk2 activity against SPAR, resembling the potency of CA-Plk2 or S299E (Fig. 4B and C). As S588A/E and S299E were all expressed at equal or lower levels than CA-Plk2 (Fig. 3B), their enhanced activity cannot be attributed simply to differences in expression. On the other hand, S299A had similar activity as WT Plk2 but was expressed at slightly lower levels, suggesting only a modest increase in activity. Taken together, these findings indicate that residues S299 and S588 are involved in autoregulation of Plk2 kinase activity.

To test whether these two phosphosites controlled Plk2 expression level or localization in neurons, myc-epitope tagged constructs were transfected into primary cultured hippocampal neurons, followed by immunocytochemistry against the recombinant protein. We found expression of each Plk2 protein to be highest in the soma and proximal dendrites with expression levels dropping dramatically with increasing distance from the soma (Fig. 5). There were no obvious alterations in subcellular localization, with each construct able to fill dendritic spines (Fig. 5A–G), and no significant differences in expression level of each mutant relative to WT-Plk2 (Fig. 5H).

To determine the effects of S299 and S588 phosphosite-mutant pairs on endogenous SPAR, cultured hippocampal neurons were cotransfected with green fluorescent protein (GFP) and WT or mutant Plk2. After 24 hr, endogenous SPAR was quantified by immunocytochemistry (Fig. 6A and B). SPAR protein was distributed in a punctate pattern along dendrites and colocalized with dendritic spines as defined morphologically by coexpressed GFP fill, consistent with its known role as a spine enriched protein associated with excitatory synapses (Fig. 6A). As expected, in control transfections, SPAR levels were highest in neurons transfected with KD-Plk2, reduced in neurons transfected with WT-Plk2, and nearly absent in neurons transfected with CA-Plk2 (Fig. 6A and B). Mirroring the results in COS7 cells, neurons transfected with S299A exhibited SPAR levels equivalent to WT-Plk2, while levels of SPAR were decreased to a much greater extent by S299E. Similarly, SPAR levels were significantly decreased relative to WT Plk2 in both S588A and S588E expressing neurons (Fig. 6A and B). These results were not due to differences in construct expression as shown above (Fig. 5). Thus, the S299E, S588A, and S588E Plk2 mutants were hyperactive against SPAR in neurons as well as in heterologous cells.

Plk2 phosphorylation site mutants regulate spine stabilization in primary hippocampal neurons

Enhanced degradation of SPAR, a spine-stabilizing protein, should lead to increased dendritic spine destabilization and/or loss. To test this possibility, we performed quantitative spine analysis in the same neurons as above using GFP as a neuronal fill. As expected, compared to the abundant spines observed in KD-Plk2 expressing cells, WT-Plk2 neurons had fewer spines, while CA-Plk2 had very few spines (Fig. 7A–C, quantified in H). S299A neurons displayed spines similar in linear density to WT-Plk2, whereas S299E, S588A, and S588E patterns resembled the sparsely distributed spines of CA-Plk2 (Fig. 7D–H). Therefore, the new hyperactive mutants were also equivalent to CA-Plk2 in downstream functional readouts of spine elimination.

DISCUSSION

In this study, we have identified several phosphorylation sites within Plk2. The sites were uncovered by unbiased mass spectrometric analysis using active Plk2 compared to inactive Plk2 in mammalian cells. There was no obvious similarity among the autophosphorylation sites, and for the most part these sites did not conform to proposed Plk2 consensus sequences (Johnson et al., 2007). However, such consensus sequences are not stringently defined, and consist merely of flanking acidic residues. Thus, the new sites shown in this work may reveal additional sequence utilization. It is also formally possible that phosphorylation of the sites identified here could have been promoted indirectly by a Plk2-dependent signaling cascade in heterologous cells. However, unequivocal demonstration that Plk2 does autophosphorylate these sites directly requires *in vitro* kinase assays using purified kinase and a modified Plk2 substrate that lacks all other potential phosphorylation sites. Such assays are difficult due to the large number of sites, and the potential requirement of priming of one site by phosphorylation at a previous site (Seeburg et al., 2008). Regardless of direct phosphorylation, these sites are novel and important for regulation of Plk2 function.

In general, our analysis showed that the majority of phosphosites did not have major effects on Plk2 conformation as only one of the mutations (S588E) resulted in a shift to the higher migrating band similar to CA-Plk2 (Fig. 3A, lanes 16 and 22). The mutations did, however, have significant effects on apparent protein stability in COS7 cells. Because all constructs were expressed from the same promoter and untranslated regions, changes in expression were likely due to alterations in protein half-life. This conclusion is supported by data showing that half-life determination with cycloheximide produces stability changes that

correspond with steady-state levels of selected mutants compared to WT Plk2, although we cannot exclude the possibility that coding region mutations could alter translational efficiency as well. There are several mechanisms that could account for changes in protein stabilization. Plk2 levels in the cell are thought to be regulated by ubiquitin-mediated degradation by the proteasome. Phosphorylation induced ubiquitination has been demonstrated for many proteins (Hunter, 2007) and all of the mutant sites we identified surrounding the PBD follow a similar pattern of non-phosphorylatable mutants increasing stability and phosphomimetic mutants resulting in protein levels similar to WT, suggesting these phosphorylation sites may be regulating the activity of a phosphodegron. In addition to phosphorylation up-regulating ubiquitination, it can also result in the opposite, decreasing ubiquitination and increasing stability (Dan et al., 2004). This is consistent with the S299A mutation decreasing protein stability in COS7 cells while the S299E mutation increases its stability relative to wild-type.

Two other mechanisms may account for the 5 phosphosites that resulted in increased stability whether they were mutated to alanine or glutamic acid. One of these sites, T413 is in a PEST motif (Zimmerman and Erikson, 2007). PEST motifs are found in many unstable proteins and have been shown to serve as targets for calcium-activated proteases and calpains as well as by the ubiquitin-proteasome pathway (Dice, 1987, Rechsteiner and Rogers, 1996, Tompa et al., 2004). Indeed, the PEST motif around T413 also contains a calpain cleavage site at Q418 as predicted from the CaMPDB calpain cleavage algorithm. A mutation of any kind in this motif may result in disruption of the degradation mechanism and increased stability of the protein. Finally, it is also possible that sites for recognition by E3 ligases may be dependant on conformational changes induced by phosphorylation rather than the negative charge introduced alone. In such a case, mutation to either alanine or glutamic acid may result in an inability to form the correct conformation to expose the E3 ligase site.

These data suggest that Plk2 autophosphorylation is an important component of its protein stability in COS7 cells. The fact that one of the hyperactive mutants exhibits similar stability to WT-Plk2 (S588E) while other hyperactive mutants like S299E and CA-Plk2 are more stable than WT suggests that phosphorylation is not exclusively a signal for degradation. There may be a combinatorial effect of multiple phosphorylations with some of the sites masking or overriding the destabilizing effects of other phosphorylated sites. Notably, when expressed in neurons, the significant differences in protein expression between WT-Plk2, KD, CA, S299A, and S588A were no longer present, suggesting that the mechanisms regulating Plk2 stability in neurons may be different than in COS7 cells, possibly reflecting the widely differing roles for Plk2 in dividing and non-dividing cells.

In addition to alterations in protein stability, several mutants strongly stimulated Plk2 kinase activity. These sites were found in two distinct loci and appeared to operate by three different mechanisms, based on their gel migration patterns, which we have used as an index of conformational state. The kinase appeared to exist in either a “closed” and detergent-resistant inactive state, or in an “open” and active state. One site, S588, was found to reside in the C-terminal region between the conserved polo boxes. Because the polo boxes are thought to form an inhibitory intramolecular loop (Jang et al., 2002a, Lowery et al., 2005), S588 could be involved in helping mediate association between PBD and kinase domain. The S588E mutation could then destabilize this association, resulting in an active and open conformation analogous to the CA-Plk2 mutation T236E (Fig. 8B). Why then does S588A also activate Plk2? It is possible that the serine residue itself is important in helping to mediate PBD-kinase domain interaction, and that replacement of this amino acid to alanine partially destabilizes the intramolecular complex to form a “loose” complex (Fig. 8C). Although this mutant did not exhibit gel mobility shift, suggesting the lack of a large

conformational change, the intramolecular association may be loosened enough to allow more rapid interconversion to the fully active, open configuration in the presence of a substrate. Interestingly, this site was one of two phosphosites also detected in Plk3 (Fig. 1C; S552 in Plk3 numbering), suggesting a conserved mechanism.

The second site that exhibited strong activation of Plk2 was S299, which resides within the kinase domain itself. Although S299A had no observable effect, behaving much like WT-Plk2 in our assays, S299E hyperactivated Plk2 without changing its gel migration, suggesting lack of major conformational changes. It is possible that S299E also partially destabilizes the kinase-PBD intramolecular interaction, similar to S588A, leading to a “loose” complex that is more easily activated by substrate (Fig. 8D, left). Alternatively, as this mutation is in the kinase domain itself, it could exert a direct effect on the kinase active site that may enlarge the cavity and allow substrate entry despite intact PBD-kinase binding (Fig. 8D, right).

It should be noted that the functional significance of the identified sites could also be important in other contexts not assayed here, such as kinase activity against different classes of substrates, or targeting to specific subcellular locales when expressed at endogenous protein levels. Of the mutations that did have a strong effect on Plk2 kinase activity, we did not find any that rendered the kinase inactive, suggesting that Plk2 does not autoregulate itself in a negative feedback manner. Rather, we speculate that Plk2 may be downregulated primarily at the level of degradative clearance by the ubiquitin-proteasome system (Pak and Sheng, 2003). However, when expressed in hippocampal neurons, the identified hyperactive mutants behaved as predicted, causing profound loss of SPAR protein and dendritic spines, similar to CA-Plk2. These results strongly indicate that these mutations are indeed functionally equivalent to a constitutively active kinase.

Conclusions

Taken together, these results reveal novel modes of intramolecular kinase regulation of Plk2. The multiple mechanisms of kinase self-activation described here suggest that once expressed, Plk2 maintains itself in a continuously active state by positive feedback amplification, perhaps until it becomes ultimately degraded. Because of the diverse roles of Plks in neurons and proliferating cells, these results may have relevance for understanding neuronal signaling events as well as cell cycle control.

Highlights

- Active, but not kinase-dead, forms of Plk2 are highly phosphorylated.
- Mutating Plk2 phosphorylation sites demonstrates regulation of protein stability.
- Plk2 phosphorylation sites regulate kinase activity towards Plk2 substrate SPAR.
- Plk2 phosphosite mutants regulate dendritic spine stability in hippocampal neurons.

Abbreviations

Plks	Polo-like kinases
PBD	polo box domain

SPAR	Spine associated Rap-GAP
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
LC-MS/MS	Liquid chromatography tandem mass spectrometry
DIV	days in vitro
WT	wild-type
KD	kinase-dead
CA	constitutively active
GFP	green fluorescent protein

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Figure 1. Identification of Plk2 autophosphorylation sites

(A) Left, comparison of SDS-PAGE gel mobility and steady-state protein level of myc epitope-tagged Plk2 kinase dead (K108M), wild-type (WT), and constitutively active (T236E) forms expressed in COS7 cells. Right, Plk2 (K108M) or (T236E) were immunoprecipitated using myc agarose and visualized on SDS-PAGE with Coomassie staining for mass spectrometric analysis. Schematic illustrates proposed open and closed conformations of differently migrating species. (B) Trypic peptides identified by LC-MS/MS with potential phosphorylation sites denoted by red and asterisk. Amino acid (aa) residue of sites is also indicated. The @ symbol next to methionines indicates that the methionine is oxidized (16 mass units higher than expected), which readily occurs during the process of running and staining/destaining the gel. (C) Positions of potential phosphorylation sites within the primary structure of Plk2 (arrows). Positions of kinase-dead (K108M, red) and constitutively active (T236E, green) mutants are indicated with lines. Sites identified in Plk3 are shown at bottom (blue) aligned with homologous residues in Plk2 (but designated with amino acid numbering for Plk3).

Figure 2. Alignment of Plk family members and location of phosphorylation sites
Numbering in amino acid residues. Red boxes indicate known phosphorylation sites as annotated at right of sequences.

Figure 3. Plk2 phosphosite mutant expression in COS7 cells

(A) Expression and gel mobility of myc epitope-tagged phosphosite mutants and KD-, WT-, and CA-Plk2 controls. Lysates from COS7 cells transfected as indicated at top of blots were analyzed by SDS-PAGE and immunoblotted with myc antibodies. Cotransfection of GFP demonstrates equivalent transfection efficiency and loading. (B) Quantification of data in (A); $n = 4-14$ replicates per construct. (C) Example immunoblots of WT and selected Plk2 mutants expressed in COS7 cells for 24 hrs and treated with cycloheximide for the indicated times. The calculated half-life from (D) is indicated to the right. (D) Half-life determination of Plk2 constructs from (C), plotted as percent remaining protein on a log scale against time ($n = 3-6$ replicates per time point per construct). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.005$.

Figure 4. Plk2 phosphosite mutant effects on kinase activity

(A) Immunoblot of the Plk2 substrate SPAR (tagged with myc-epitope) cotransfected with each phosphosite mutant and KD, WT, and CA-Plk2 controls. Mutant pairs S299A/S299E and S588A/S588E exhibited differential levels of SPAR between the non-phosphorylatable and phosphomimetic mutants. (B) Representative blot of Plk2 controls and 4 phosphosite mutants identified in (A) cotransfected with myc-SPAR for quantification. (C) Quantification of SPAR cotransfected with Plk2 controls and phosphosite mutants; $n = 3-4$ replicates per condition; * $p < 0.05$ and ** $p < 0.005$ relative to SPAR cotransfected with WT-Plk2.

Figure 5. Expression of phosphosite mutants in primary hippocampal neurons
 (A–G) Cultured hippocampal neurons (DIV 16) were transfected with constructs as indicated and immunostained 24 hr post-transfection with an anti-myc antibody to visualize exogenous Plk2. Scale bar in (A) represents 20 μ m. (H) Quantification of data from A–G.

Figure 6. Effects of phosphosite mutants on endogenous SPAR expression

(A) DIV 16 hippocampal neurons were cotransfected with GFP and phosphosite mutants or controls as indicated. After 24 hrs, neurons were fixed and immunostained with anti-GFP to identify transfected cells and with anti-SPAR antibodies to visualize endogenous SPAR. Representative dendrites are enlarged below each neuron image. White arrows indicate SPAR puncta that colocalize with GFP-filled spines. (B) Quantitation of data from (A); $n = 7-11$ neurons per transfection; * $p < 0.05$ and ** $p < 0.005$ relative to SPAR in neurons transfected with WT-Plk2. Scale bar represents 20 μm .

Figure 7. Effects of phosphomutants on dendritic spines

(A–G) DIV 16 hippocampal neurons were cotransfected with GFP and phosphosite mutants or controls as indicated. After 24 hrs, neurons were fixed and immunostained with anti-GFP antibodies. Images were captured on a laser scanning confocal microscope for analysis of spine density. Representative dendrites are enlarged below each neuron image. (H) Quantification of data in (A–G); $n = 5–8$ neurons per group; * $p < 0.05$ relative to WT-Plk2. Scale bar in (A) represents 20 μm.

Figure 8. Hypothetical models for Plk2 autophosphorylation regulation of kinase activity
 (A) When inactive, the polo box domain (PBD) consisting of polo boxes (PB) 1 and 2 forms an intramolecular inhibitory loop that creates a conformationally closed state and allosterically blocks the enzymatic active site. (B) Activation of Plk2 by mutants (T236E or S588E) disrupts PBD-kinase domain interactions to form an open, active state. (C) S588A mutation weakens but does not fully disrupt PBD-kinase interaction, resulting in a conformationally closed but "loose" state that may be more easily activated in the presence of substrate. (D) S299E mutation may result in a similar "loose" and closed conformation similar to S588A (left), or may directly cause allosteric opening of the enzymatic active site (right) to increase kinase activity. In contrast, S299A mutation has no apparent effect and is functionally wild-type (not shown).