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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. **© 2011 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: 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 spine-associated Rap-GAP (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. 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 composed of one or two polo boxes, binding modules important for interactions with target substrates and for proper subcellular localization (Elia et al., 2003a,b). 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 Weerdt 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 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

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Abbreviations: CA, constitutively active; CaMPDB, Calpain for Modulatory Proteolysis Database; DIV, days *in vitro*; GFP, green fluorescent protein; KD, kinase-dead; LC-MS/MS, liquid chromatography tandem mass spectrometry; PBD, polo box domain; Plks, polo-like kinases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPAR, spine-associated Rap-GAP; WT, wild-type.

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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, Solon, OH, USA), rabbit anti-Myc antibody (Cell Signaling, Danvers, MA, USA) (Pak and Sheng, 2003), rabbit anti-SPAR antibody (Pak et al., 2001). Alexa Fluor 488 and 555 (Invitrogen, Carlsburg, CA, USA) were used as secondary antibodies for all immunofluorescence.

Cell culture and transfection

COS7 cells (ATCC, Manassas, VA, USA) were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 0.1% gentamicin (Invitrogen). COS7 cells were transiently transfected with 1 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions. After expression for 24 h, cells were harvested in 1$\times$ SDS loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% Bromophenol Blue). For cycloheximide experiments, 216 μM cycloheximide was added to COS7 cells 16 h after transfection with the different Plk2 constructs and harvested as mentioned previously at the indicated time-points. To determine protein half-life, data were 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 ($\sim$150 cells/mm^2) on coverslips coated with poly-D-lysine (Sigma, St. Louis, MO, USA) and laminin (2 μg/ml, Roche, Basel, Switzerland). Cultures were grown in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM glutamine, and 12.5 μM glutamate. Neurons were transfected at DIV 16 using Lipofectamine 2000 (Invitrogen). For transfection with Lipofectamine 2000, 2 μg of DNA in 25 μl serum-free Neurobasal media (Invitrogen) was mixed with 2 μl of Lipofectamine 2000 in 25 μl 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 h before fixing.

Immunoblotting

Samples were separated by SDS-PAGE, transferred to nitrocellulose, and blocked with 5% nonfat dry milk in 1$\times$ 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 h at room temperature. Blots were imaged through enhanced chemiluminescence system (Western Lightning (Perkin Elmer, Waltham, MA, USA) or SuperSignal West Femto (Pierce, Rockford, IL, USA). 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 h 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 h at room temperature.

Quantification and image analysis

Microscopy images were acquired using an Axiovert 200M epifluorescence inverted microscope (Zeiss, Thornwood, NY, USA) 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, Sunnyvale, CA, USA). 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, NY, USA) 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

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