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Identification of High Affinity Polo-like Kinase 1 (Plk1) Polo-box Domain Binding Peptides Using Oxime-Based Diversification

Fa Liu,[†,⊥] Jung-Eun Park,[‡,⊥] Wen-Jian Qian,[†] Dan Lim,[§] Andrej Scharow,[∥] Thorsten Berg,[∥] Michael B. Yaffe,[§] Kyung S. Lee,*[,‡] and Terrence R. Burke, Jr.*[,†]

[†]Chemical Biology Laboratory, Molecular Discovery Program, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, Maryland 21702, United States

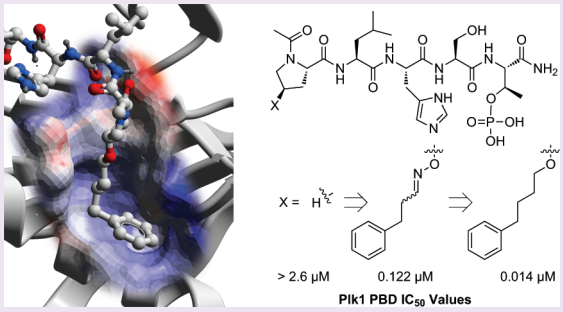
[‡]Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, United States

[§]Department of Biology and Biological Engineering, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, United States

[∥]Institute of Organic Chemistry, University of Leipzig, Leipzig, Germany

Supporting Information

ABSTRACT: In an effort to develop improved binding antagonists of the polo-like kinase 1 (Plk1) polo-box domain (PBD), we optimized interactions of the known high affinity 5-mer peptide PLHSpT using oxime-based post solid-phase peptide diversification of the *N*-terminal Pro residue. This allowed us to achieve up to two orders of magnitude potency enhancement. An X-ray crystal structure of the highest affinity analogue in complex with Plk1 PBD revealed new binding interactions in a hydrophobic channel that had been occluded in X-ray structures of the unliganded protein. This study represents an important example where amino acid modification by post solid-phase oxime ligation can facilitate the development of protein−protein interaction inhibitors by identifying new binding pockets that would not otherwise be accessible to coded amino acid residues.



Members of the polo subfamily of protein kinases (collectively, Plks) play pivotal roles in cell proliferation. Among them, Plk1 has been studied most extensively because of its ability to promote tumorigenesis in human cells.[1] Plk1 offers two distinct drug targets within one molecule: an *N*-terminal catalytic domain and a *C*-terminal polo-box domain (PBD). Over the years, a great deal of effort to generate anti-Plk1 inhibitors has been focused on targeting the catalytic domain. However, a large body of evidence suggests that the PBD serves as a cis-acting phospho-binding module that is essentially required for bringing the catalytic activity of Plk1 to its binding targets at specific subcellular locations.[2−4] Thus, inhibition of PBD-dependent Plk1 function could serve as an attractive alternative that is worthwhile to explore.

We have shown that post solid-phase peptide diversification by introducing aminooxy functionality at either the 3- or 4-position of a Pro pyrrolidine ring and subjecting the resulting peptides to oxime-based ligation reactions can have significant utility in the study of protein−protein interaction (PPI) inhibitors.[5−7] The chemical stability of oximes[8] and the ease with which they can be prepared in high purity make oxime ligation an attractive method for the post solid-phase construction of peptide libraries that can be biologically evaluated directly without purification.[5−7] Oxime ligation can be a highly effective way to explore peptide−protein interactions in ways that are not possible using coded amino acids.

The polo-box interacting protein 1 (PBIP1) is a known Plk1 substrate that undergoes phosphorylation at T78 to form a Plk1 PBD-binding ligand.[9] The PBIP1-derived 5-mer peptide 74-PLHSpT-78 (**1**) represents a minimal sequence that specifically interacts with the Plk1 PBD with high affinity (K_d = 0.45 μM), but not with the two closely related Plk2 and Plk3 PBDs.[10] Co-crystal structures of the Plk1 PBD in complex with short phosphopeptides[3,4,10,11] show that while interaction of the SpT motif is similar in all of these structures, for PLHSpT (PDB id 3HIK) and PPHSpT (PDB id 3C5L) the *N*-terminal Pro residues bind in greatly different orientations.[10] The combined protein binding surface encompassing these Pro residues covers a broad region that seemed to afford an ideal opportunity for oxime-based post solid-phase ligation originating from the *N*-terminal Pro residue (Suporting Information Figure S1). Herein we report an application of this approach to explore structural variations originating from the Pro residue, which

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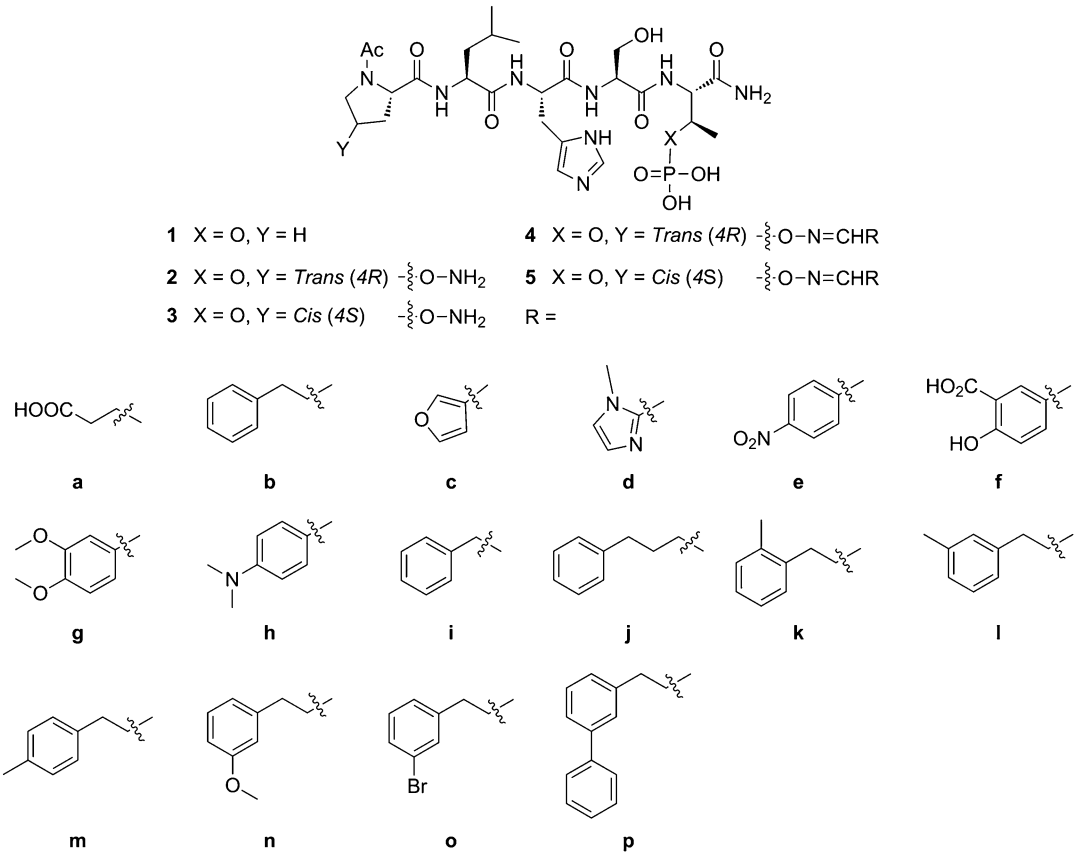

Figure 1. Structures of peptides and reagents used in the generation of oxime libraries.

resulted in the identification of analogues that exhibit greater than two orders of magnitude enhanced Plk1 PBD affinities.

Oxime libraries were prepared to diversify the Pro residue of peptide **1**. The pyrrolidine ring of the original residue was maintained, since biochemical data indicate that this Pro residue is important both for Plk1 PBD-binding affinity and specificity relative to the closely related kinases Plk2 and Plk3.[10] Fmoc-based solid-phase peptide synthesis employing orthogonally protected *trans* (4*R*) and *cis* (4*S*) aminooxyproline derivatives[5] provided the corresponding peptides **2** and **3**, respectively, bearing free aminooxy groups at the 4-position of the Pro residues (Figure 1). Oxime-containing peptide libraries (**4** and **5**) were then prepared by conjugating peptides **2** and **3** with a set of 8 selected aldehdyes (**a**−**h**). The choice of these aldehydes was based on their diversity (charge, size, aromatic, aliphatic, heteroaryl, *etc.*). The resulting peptides were directly evaluated for their ability to compete with an immobilized PBIP1-derived p-T78 peptide in ELISA-based Plk1 inhibition assays using HEK 293A cell lysates expressing GFP-HA-fused Plk1 (Supplementary Figure S6).[10] The peptides containing oximes formed from 3-phenylpropanal (**4b** and **5b**) showed the greatest affinity enhancement relative to the parent **1**, with **4b** being slightly more potent than **5b**.

More focused libraries were then prepared to explore structural features of **4b** and **5b**. Decreasing (**4i** and **5i**) or increasing (**4j** and **5j**) the alkyl chain length by one methylene group decreased affinity with the *trans* (4*R*) isomers (**4i** and **4j**) being slightly more potent than the corresponding *cis* (4*S*) isomers (**5i** and **5j**) (Supplementary Figure S7). A methyl scan (**4k**−**4m**) conducted on the phenyl ring of **4b** had little effect on binding affinity, although substitution at the *meta*-position (**4l**) slightly increased affinity (Supplementary Figure S8). Based on the higher affinity of **4l**, additional *meta*-substituted analogues (**4n**−**4p**) were prepared, and all of these exhibited binding potencies similar to **4b**, although the 3-methoxy substituent (**4n**) slightly increased the binding affinity, while the 3-phenyl substituent (**4p**) slightly decreased the affinity. Replacing the oxime functionality of **5b** with an amidooxy group (**6**, Figure 2a) was also deleterious.

The analogues **4b** and **5b** were converted to the corresponding 4-phenylbutyl ethers (**7** and **8**, respectively, Figure 2a) using preformed proline derivatives. The ethers removed the imine portion of the original oximes, while maintaining a similar chain extension. Both **7** and **8** had significantly higher binding affinities than their corresponding oximes, with the *trans* (4*R*) isomer (**7**) being more potent than the *cis* (4*S*) epimer (**8**) (Supplementary Figure S8). Binding was phospho-dependent, since a near total loss of affinity was observed for the variant in which the phosphothreonine residue had been replaced by a threonine residue (**7(pT5T)**, Supplementary Figure S9). Additionally, in order to exclude possible inhibition by nonspecific "promiscuous" mechanisms arising from hydrophobicity that are unrelated to specific interactions with PBD,[12] we made use of the fact that the "SpT" dipeptide motif is critical for high affinity Plk1 PBD-binding and that replacement of the serine residue by an alanine (S/A replacement) typically abrogates binding.[4] We observed that S/A variants, **7(S4A)** and **8(S4A)**, showed a significant loss of affinity relative to the corresponding parent peptides (Supplementary Figure S9). This argued strongly that binding of **7** and **8** was specific in nature.

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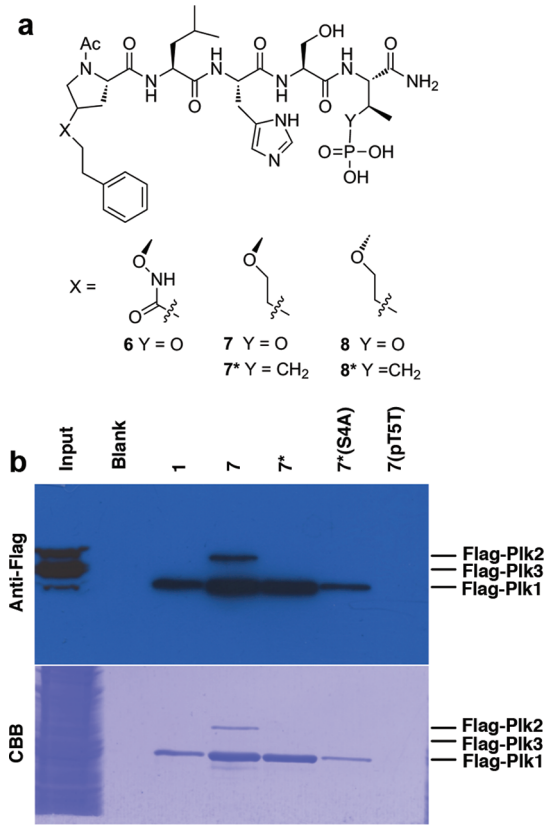

Figure 2. (a) Structures of peptides discussed in the text. (b) Peptide pull-down assay showing ability of the indicated peptides (top) to pull down Plk1−3 from mitotic 293T cell lysates expressing kinase-inactive Flag-Plk1 (K82M), Flag-Plk2 (K108M), or Flag-Plk3 (K52R) as described in the Methods. Top: visualized using anti-Flag antibodies. Bottom: visualized using Coomassie staining (CBB). Input, 5% of total lysates used for pull-downs. Locations of relevant protein bands are shown.

The ELISA-based Plk1 inhibition data (Supplementary Figures S6−S9) provided relative binding affinities that served to guide structural modifications. In order to quantitate the binding affinities of selected analogues, the assays were repeated using an expanded range of concentrations (Supplementary Figure S10). This allowed an estimation of IC_{50} values: **1** (20 μM); **4b** (0.43 μM); **7** (0.04 μM); **7*** (0.20 μM), and **7(S4A)** (43 μM) [where **7*** indicates replacement of the pT residue with (2*S*,3*R*)-2-amino-3-methyl-4-phosphonobutyric acid (Pmab) as a phosphatase-stable pT mimetic[13]].

Binding affinities were also determined independently using fluorescence polarization techniques, which measured the ability of peptides to compete with a 5-carboxyfluorescein-labeled variant of the peptide GPMQSpTPLNG-OH (**9**) (5-CF-**9**) for binding to purified Plk1 PBD protein (Table 1).[14] In this latter assay, the WT 5-mer parent peptide **1** (40% ± 2% inhibition at 2.56 μM concentration) was slightly less potent than the control 10-mer peptide (**9**, IC_{50} = 1.12 ± 0.26 μM). The isomeric oximes **4b** and **5b** were approximately an order of magnitude more potent than **1** (IC_{50} = 0.122 ± 0.024 μM and 0.433 ± 0.083 μM, respectively). Consistent with the ELISA-based inhibition assay, the *trans*-isomer bound with higher affinity than the *cis*-isomer. Conversion of the oximes **4b** and **5b** to their corresponding ether analogues was accompanied by another order of magnitude increase in affinity (**7**, IC_{50} = 0.014 ± 0.001 μM and **8**, IC_{50} = 0.038 ± 0.009 μM), which

Table 1. Plk1 PBD-Binding IC_{50} Values[a]

no.	IC_{50} [μM]
1	*b,c*
4b	0.122 ± 0.024
5b	0.433 ± 0.083
7	0.014 ± 0.001
7(S4A)	4.15 ± 0.96
7*	0.086 ± 0.017
7*(S4A)	*c,d*
8	0.038 ± 0.009
8*	0.114 ± 0.003
9	1.12 ± 0.26

[a]Determined by competition against binding of 5-carboxyfluorescein-GPMQSpTPLNG-OH (5-CF-**9**) and the Plk1 PBD as determined by fluorescence polarization assays. [b]40 ± 2% inhibition at 2.56 μM. [c]Autofluorescence-limited. [d]45 ± 7% inhibition at 2.56 μM.

represents an approximate two orders of magnitude enhancement relative to the WT parent peptide **1**. The Pmab-containing versions of **7** and **8** bound with less affinity than their pThr-containing parents. This was observed for both **7** (**7***, IC_{50} = 0.086 ± 0.017 μM; 6-fold less potent) and **8** (IC_{50} = 0.038 ± 0.009 μM as compared to **8***, IC_{50} = 0.114 ± 0.003 μM; 3-fold less potent).

We introduced onto **7** and selected variants, *N*-terminal Cys residues tethered by *n*-hexanoylamide chains and covalently conjugated the resulting peptides to SulfoLink Coupling Gel. We then measured the relative abilities of these preparations to bind to Plk1, Plk2, or Plk3, when exposed to lysates of mitotic 293T cells containing Flag-fused kinase dead forms of Plk1 (K82M), Flag-Plk2 (K108M), or Flag-Plk3 (K52R) (Figure 2b). While confirming our previous findings that **1** is highly specific for Plk1,[10−15] a faint band corresponding to binding of peptide **7** to Plk2 was observed in addition to a very intense band associated with its binding to Plk1. A Plk2 band was not seen for the Pmab-containing analogue **7***, although more than 200-fold and approximately 6-fold reduced Plk1 PBD binding affinities of **1** and **7*** relative to **7** could render binding of these peptides to Plk2 too faint for detection.

In order to determine the molecular basis for the enhanced binding affinity of **7**, we solved the X-ray co-crystal structure of **7** in complex with Plk1 PBD (Supplementary Table S3 and Figure S12). The "HSpT" residues of **7** were nearly superimposable with those of the PBD-bound **1** in the 3HIK structure (Figure 3a). However, significant structural differences were observed with the Leu residue, where the psi angle (ψ = −1.9° and 157.9° for **1** and **7**, respectively) placed the adjacent *N*-terminal Pro residues in nearly opposing directions (Figure 3a). This is reminiscent of what is observed in PBD-bound "PPHSpT" (PDB id 3C5L), where the latter's P2 psi angle (ψ = 145.4°) places its P1 residue in a similar position as **7** (compare with Supplementary Figure S1). In this new orientation, the *trans*-(4*R*) phenylbutyloxy substituent on the P1 pyrrolidine ring of **7** is directed across the β2 and β3 sheets of PBD, where it terminates with its phenyl ring nestled against the αB helix. The net effect is to reveal a channel that is occluded in the binding of parent peptide **1** (compare Figure 3, panels b and c).

The interactions of the -$(CH_2)_4$-Phe moiety of **7** are superimposable with those of a recently reported -$(CH_2)_8$-Phe adduct originating from the histidine imidazole ring of **1** (peptide **10**, PDB id 3RQ7) (Figure 3d).[15] The binding of the

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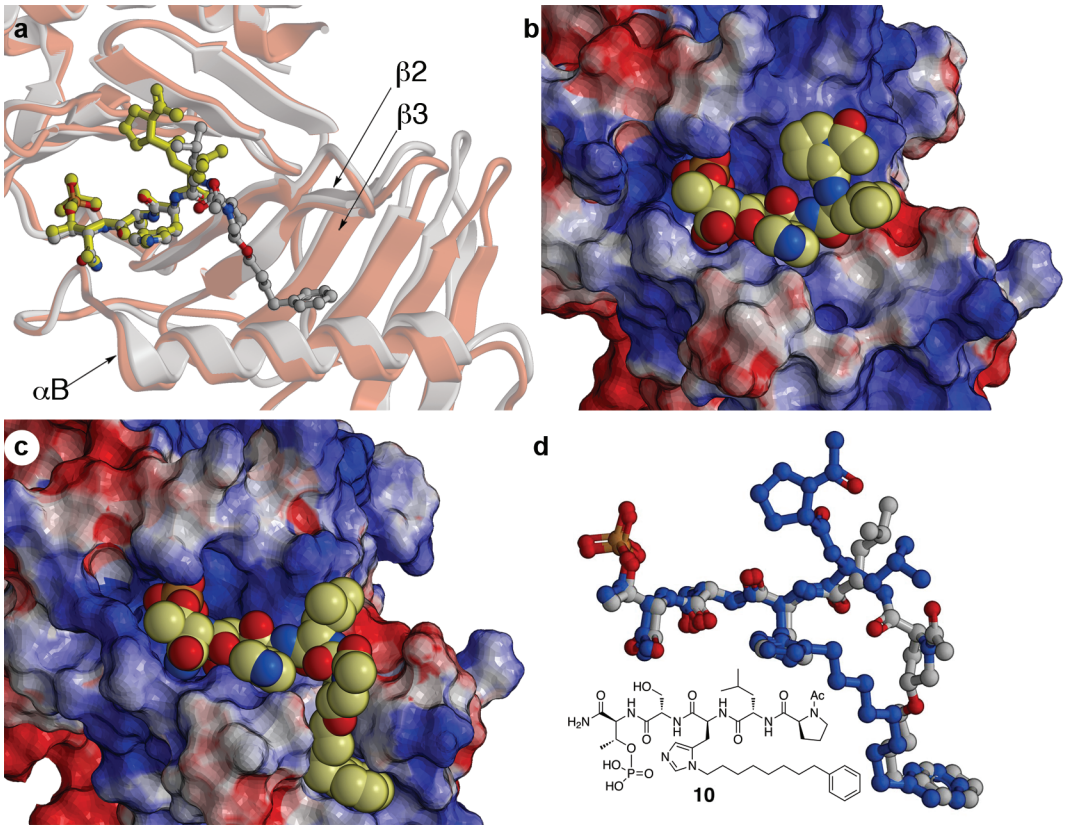

Figure 3. X-ray co-crystal structures of Plk1 PBD complexed with peptides **1** and **7** and **10**. (a) PBD in complex with **1** (PBD id 3HIK; protein backbone and peptide shown in red) superimposed on the complex with **7** (protein backbone in gray with peptide **7** colored by atom). Protein structural features are labeled as indicated in reference 3. (b, c) Electrostatic surfaces of PBD in complex with **1** (b) and **7** (c) with coloring based on an arbitrary electrostatic potential scale (positive = blue; negative = red). Peptides are rendered as CPK and colored by atom (blue = nitrogen; yellow = carbon; tan = phosphorus; and red = oxygen). (d) Superposition of PBD-bound peptides **7** (carbons in gray) with **10** (PDB id 3RQ7) (colored blue) showing overlap of alkylphenyl chain functionalities.

phenyl ring of **7** was also similar to what was recently reported for the phenyl ring in the F1 residue of "FDPPLHSpTA" (peptide **11**, PDB id 3P37) (Supplementary Figure S13).[16] Peptide **11** represents an extended version of **1** encompassing residues 71−79 of the T78 region within the PBIP1 protein.[9] For PBD complexes of **7**, **10**, and **11**, important protein residues lining the binding channel (V415, Y417, Y421, L478, Y481, F482, and Y485) provide a highly hydrophobic environment (Supplementary Figure S13 and Table S4). Hydrophobic interactions with the residues within the channel are similar for **7**, **10**, and **11**, with the exceptions that **11** has significantly more contact with Y417 (due to face-on binding with the peptide P3 residue) and **7** and **10** make greater contact with the L478 residue than **11** does. These residues are oriented similarly to those found in the PBD·**1** complex (PDB id 3HIK), with a critical exception arising from the Y481 side chain, which occludes the binding channel in the unliganded protein (PDB id 1Q4O) and in the PBD·**1** complex. Dramatic rotation of the Y481 side chain is required to reveal the binding channel and open access to the αB helix-proximal aryl-binding pocket in the PBD complexes of **7**, **10**, and **11** (Supplementary Figure S14).[15,16]

By application of a facile oxime-based post solid-phase peptide diversification protocol, we have realized a 2 orders of magnitude improvement in affinity over that of the starting WT peptide. This was achieved while maintaining good Plk1 PBD selectivity relative to the PBDs of Plk2 and Plk3. More importantly, an X-ray co-crystal structure of the most potent analogue revealed new binding interactions in a hydrophobic channel of the PBD that is occluded in the unliganded protein or in the protein complexed with parental 5-mer peptide . Our work reinforces the value of oxime-based post solid-phase ligation for the discovery of new binding interactions that would not otherwise be apparent from the binding of peptides containing naturally encoded amino acids. Viewed as a "tethered fragment" approach, oxime ligation has distinct advantages over traditional fragment-based screening, where the low binding affinities of fragment libraries present inherent limitations.[17] The potential power of the directed approach employed in the current study is shown by its ability to identify binding interactions highly similar to those previously identified through serendipity. Our findings expand the design parameters available for development of Plk1 PBD-binding antagonists, and provide direction that may be useful for optimizing PPI inhibitors in general.

METHODS

Peptide Synthesis. Peptides were synthesized and purified as described in the Supporting Information.

Post Solid-Phase Diversification: Preparation of Oxime-containing Peptides 4a−p and 5a−p (5 mM in DMSO). A mixture of HPLC-purified aminooxy-proline containing peptide (**2** or **3**) (15 mM in DMSO, 10 μL), aldehyde (**a**−**p**, Supplementary Figure S2) (15 mM in DMSO, 10 μL) and acetic acid (70 mM in DMSO, 10 μL) was gently agitated at RT (overnight). Crude reaction mixtures

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were used directly for biological evaluation. **4b** and **5b** were scaled up and purified by preparative HPLC.

ELISA-Based PBD-Binding Inhibition Assays. Peptide pull-down assays were carried out essentially as described previously.[10,15] A biotinylated p-T78 peptide was first diluted with 1X coating solution (KPL Inc.) to a final concentration of 0.3 μM, and then 100 μL of the resulting solution was immobilized onto a 96-well streptavidin-coated plate (Nalgene Nunc). The wells were washed once with PBS plus 0.05% Tween20 (PBST), and incubated with 200 μL of PBS plus 1% BSA (blocking buffer) for 1 h to prevent nonspecific binding. Mitotic 293A lysates expressing HA-EGFP-Plk1 were prepared in TBSN buffer ($\sim$ 60 μg total lysates in 100 μL buffer), mixed with the indicated amount of peptide ligands and applied immediately onto the biotinylated p-T78 peptide-coated ELISA wells, and then incubated with constant rocking for 1 h at 25 °C. Following incubation, the ELISA plates were washed 4 times with PBST. To detect bound HA-EGFP-Plk1, the plates were probed for 2 h with 100 μL well^{-1} of anti-HA antibody at a concentration of 0.5 μg mL^{-1} in blocking buffer and then washed 5 times. The plates were further probed for 1 h with 100 μL well^{-1} of HRP-conjugated secondary antibody (GE Healthcare) at a 1:1,000 dilution in blocking buffer. The plates were washed 5 times with PBST and incubated with 100 μL well^{-1} of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Sigma) until a desired absorbance was achieved. The reactions were stopped by the addition of 100 μL well^{-1} of stop solution (Cell Signaling Technology) and the optical densities (OD) were measured at 450 nm using an ELISA plate reader (Molecular Devices). Data are shown in Supplementary Figures S6−S10.

PBD Fluorescence Polarization Competition Binding Assays for Plk1. Competition assays for the Plk1 PBD were also performed essentially as described.[14,18,19] In brief, 5-carboxyfluorescein-GPMQSpTPLNG-OH (5-CF-**9**) (final concentration 2 nM) was incubated with the Plk1 PBD (final concentration: 20 nM) in the presence of the test peptides (final concentrations of buffer components: 50 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.1% (v/v) Nonidet P-40 substitute, 1 mM dithiothreitol, and 2% (v/v) DMSO.) Fluorescence polarization was analyzed after 60 min. Inhibition curves were fitted using SigmaPlot (SPSS) and shown in Supplementary Figure S11. All experiments were performed in triplicate. Numerical IC_{50} values are shown in Table 1.

Peptide Pull-Down Assays. Peptide pull-down assays were carried out essentially as described previously.[10,15] To study Plk1 PBD-binding specificity, test peptides were covalently conjugated to beads using SulfoLink Coupling Gel (Pierce) via either an *N*-terminal Cys-$(CH_2)_6$-CO linker. Mitotic lysates expressing Plk1−3 were prepared from 293T cells transfected with Flag-Plk1 (K82M), Flag-Plk2 (K108M) or Flg-Plk3 (K52R) (a gift of Wei Dai, New York University School of Medicine, NY) and treated with 200 ng mL^{-1} of nocodazole for 16 h. Cell lysates were then prepared in TBSN buffer {20 mM Tris-Cl (pH8.0), 150 mM NaCl, 0.5% Np-40, 5 mM EGTA, 1.5 mM EDTA, 20 mM *p*-nitrophenylphosphate and protease inhibitor cocktail (Roche)} and incubated with bead-immobilized peptides for 2 h at 4 °C. The peptide-associating proteins were precipitated, washed, boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE), and then subjected to immunoblotting analysis with anti-Flag antibody and the enhanced chemilunimescence (ECL) detection system (Pierce). The same membrane was also stained with Coomassie (CBB). Signal intensities were quantified using Image J program. Results are shown in Figure 2b.

X-ray Crystallography of Peptide 7 in Complex with Plk1 PBD Protein. Experimental details and refinement data are provided in the Supporting Information. The graphics in Figure 3 Graphics generated using ICM Chemist Pro (v3.7) by Molsoft, Inc. (www.molsoft.com). Final graphics were assembled using Adobe Photoshop CS4.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis for the preparation of proline analogues employed in peptide synthesis and synthesis of peptides and associated analytical data; X-ray crystallographic crystallization and refinement data for Plk1 PBD complexed with **7**; Plk1 PBD ELISA binding curves; Plk1 PBD fluorescence polarization binding curves and graphics related to the Plk1 PBD binding of **1**, **7**, **10**, and **11**. NMR spectra for synthetic proline analogues and HPLC traces for peptides **2**, **3**, **4b**, **5b**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Protein Data Bank coordinates for PBD in complex with **7** have been deposited under accession code 4DFW.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: tburke@helix.nih.gov; kyunglee@mail.nih.gov.

Author Contributions

⊥These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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