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Crystallization and preliminary X-ray diffraction studies on the human Plk1 Polo-box domain in complex with an unphosphorylated and a phosphorylated target peptide from Cdc25C

Polo-like kinase (Plk1) is crucial for cell-cycle progression *via* mitosis. Members of the Polo-like kinase family are characterized by the presence of a C-terminal domain termed the Polo-box domain (PBD) in addition to the N-terminal kinase domain. The PBD of Plk1 was cloned and overexpressed in *Escherichia coli*. Crystallization experiments of the protein in complex with an unphosphorylated and a phosphorylated target peptide from Cdc25C yield crystals suitable for X-ray diffraction analysis. Crystals of the PBD in complex with the phosphorylated peptide belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 38.23$, $b = 67.35$, $c = 88.25$ Å, $\alpha = \gamma = \beta = 90^\circ$, and contain one molecule per asymmetric unit. Crystals of the PBD in complex with the unphosphorylated peptide belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 40.18$, $b = 49.17$, $c = 56.23$ Å, $\alpha = \gamma = 90$, $\beta = 109.48^\circ$, and contain one molecule per asymmetric unit. The crystals diffracted to resolution limits of 2.1 and 2.85 Å using synchrotron radiation at the European Synchrotron Radiation Facility (ESRF) and the Swiss Light Source (SLS), respectively.

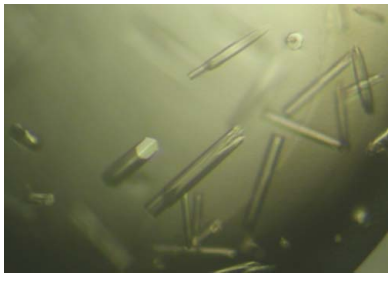
1. Introduction

Polo-like kinases (Plks) are a family of enzymes that control cell-cycle progression *via* the regulation of centrosome maturation and separation, mitotic entry, metaphase and anaphase transition, mitotic exit and cytokinesis. This tightly regulated space–time process depends on enzyme families that regulate protein phosphorylation and play a crucial role in cell-cycle progression. Plks have been identified in several species from yeast to human and are highly conserved throughout evolution (Barr *et al.*, 2004). Whereas the vertebrate species have several Plk family members (Plk1, Plk2/Snk, Plk3/Prk/Fnk and Sak), yeast and *Drosophila melanogaster* have only one. Plk1 substrates include Cdc25C phosphatase, APC/C subunits (Cdc27, Cdc16, Tsg24), cyclin B, SCC1 cohesin, mammalian kinesin-like protein 1 (MKLP-1) and other kinesin-related motor proteins (Barr *et al.*, 2004). All of them are involved in different mechanisms during mitosis.

This enzyme family is composed of a common N-terminal catalytic domain and a C-terminal regulatory domain with highly conserved sequences named Polo boxes. The Polo-box motif is only found in the Polo-like kinases and contains a characteristic sequence that is the hallmark of this protein family. This motif is likely to be involved in an auto-regulatory mechanism and in targeting the kinases to their substrates (Jang *et al.*, 2002; Lee *et al.*, 1998). The intramolecular interactions between the catalytic and the PBD domains as well as the phosphorylation of Plk1 regulate the activation of its protein kinase activity (Jang *et al.*, 2002; Liu *et al.*, 2004).

Mutations in the Plk1 PBD in *Drosophila*, *Schizosaccharomyces pombe* and *Saccharomyces cereviasiae* orthologues cause mitotic defects, including monopolar spindles, aberrant chromosome segregation and failure of cytokinesis (Simchen & Kassir, 1989; Sunkel & Glover, 1988; Ohkura *et al.*, 1995; Carmena *et al.*, 1998; Song *et al.*, 2000).

Some evidence indicates that Plk1 forms part of the regulatory circuit that controls mitosis entry though binding to phosphorylated Cdc25C by its PBD. Indeed, Plk1 can phosphorylate and thereby regulate both Cdc25C (Qian *et al.*, 1998; Kumagai & Dunphy, 1996)



and Myt1 (Nakajima *et al.*, 2003; Okano-Uchida *et al.*, 2003). This would seem to be consistent with the hypothesis that Plk1 is the 'trigger' kinase for the activation of Cdk1. However, an alternative view holds that Plk1 activation depends on the prior activation of Cdk1, in which case Plk1 would function primarily in feedback loops. Whether Plk1 regulates Cdc25C phosphatase primarily at the level of activity or localization (or both) remains to be established.

Previous structural studies of the PBD interaction with target substrates (Elia, Rellos *et al.*, 2003; Cheng *et al.*, 2003) have been performed with a non-physiological peptide. This peptide was found in an immobilized library of partially degenerate phosphopeptides during a proteomic screen looking for phospho-binding domains (Elia, Cantley *et al.*, 2003). Here, we describe the first crystals of the human PBD in complex with an unphosphorylated and a phosphorylated target peptide from its binding region in Cdc25C, a Plk1 natural substrate. We also report the expression, purification, crystallization and preliminary X-ray characterization of both protein crystals.

2. Materials and methods

2.1. Protein expression and purification

The cDNA sequence corresponding to residues 367–603 of human Plk1 was amplified by PCR and cloned into a pGEX-6P-2 (Pharmacia) vector using *Eco*RI and *Bam*HI restriction sites. *Escherichia coli* BL21(DE3) pLys cells were transformed with the vector pGEX-6P-2:PBD (Elia, Rellos *et al.*, 2003; Cheng *et al.*, 2003). Protein expression was tested in BL21, Rosetta and BL21-star strains; the BL21(DE3) plys strain was selected. Cell culture was performed at 293 K and the culture was induced with 0.1 m*M* IPTG overnight. Cells were collected in the morning and the pellets were frozen at 193 K. Upon thawing, the cells were disrupted by sonication at 277 K and cell debris was removed by centrifugation. The protein was purified using glutathione-Sepharose beads (Amersham Biosciences). The beads were washed with buffer *A* [10 m*M* HEPES pH 7.5, 200 m*M* NaCl, 3.4 m*M* EDTA and 0.01%(*v*/*v*) monothioglycerol] and buffer *A* plus 0.1% Triton X-100. The PBD-GST eluted with 20 m*M* glutathione in buffer *A*. The GST-PBD fusion was dialyzed against 50 m*M* Tris pH 7.0, 500 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT. The GST tag was cleaved with 1:10(*v*:*v*) 3C precision protease overnight at 277 K. Subsequently, the PBD was separated from the GST protein using a second glutathione-Sepharose column. The flowthrough was collected and concentrated and then loaded onto a gel-filtration column (Superdex 75, Amersham Biosciences) previously equilibrated with 20 m*M* Tris pH 8.0, 500 m*M* NaCl, 1 m*M* EDTA and 3 m*M* DTT. The protein was concentrated to 7 mg ml^{-1} using an ultrafiltration cell (Amicon), flash-frozen in liquid nitrogen and stored at 193 K. The protein homogeneity and purity was checked by SDS–PAGE (15%) gel, a native PAGE (15%) gel and an IEF gel, with optimal results. Protein concentration was determined by a dye-binding assay (BioRad) using BSA as a standard.

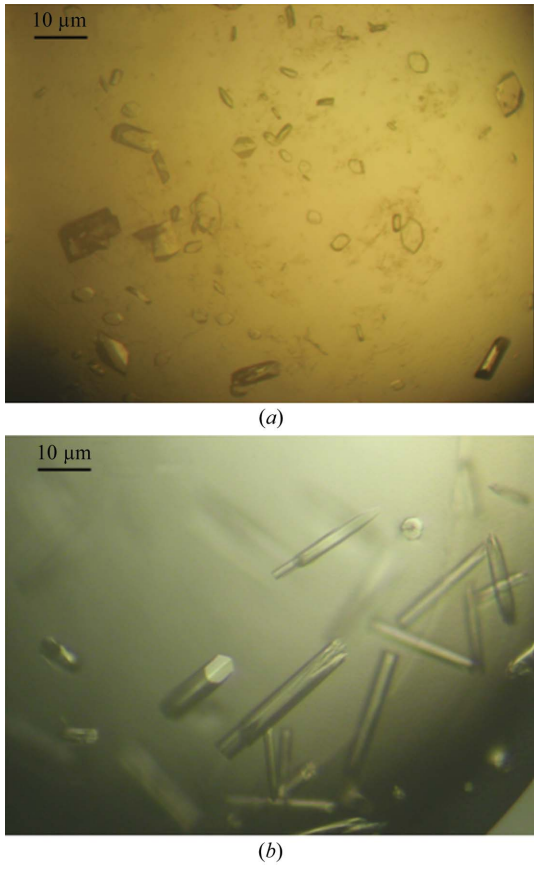


Figure 1
Crystals of Plk1 PBD in complex with Cdc25C target peptide in its phosphorylated and unphosphorylated state were grown at 289 K. (*a*) Monoclinic crystals. (*b*) Orthorhombic crystals. The human PBD crystals in complex with the peptide behaved well with the cryobuffer conditions described in the text and diffracted to 2.85 and 2.10 Å, respectively (see §3 for details). The pictures were taken at different magnification settings.

2.2. Crystallization

Crystallization screenings were performed with a Cartesian robot (Genomic Solutions) using the sitting-drop method with nanodrops of 0.2 µl. Initially, small crystals and needles were obtained after 48 h using Crystal Screens I and II from Hampton Research, CA, USA. Optimization of the crystallization conditions was consequently necessary. Improvement of the crystal quality and size was achieved by refining the initial crystallization conditions. Refinement of the crystallization conditions was performed using the hanging-drop method in Linbro plates. In both the Cdc25C-P and the Cdc25C cocrystallization assays the protein sample was in 20 m*M* Tris pH 8.0, 500 m*M* NaCl, 1 m*M* EDTA plus 3 m*M* DTT.

The Cdc25C target peptides [Cdc25C-P sequence LLCS(pT)-PNGL, Cdc25C sequence LLCSTPNGL] were synthesized (Genosphere Biotechnologies) and checked by mass spectrometry. The concentrations of the peptide samples were measured by one-dimensional proton NMR, comparing the intensity of the peptide methyl signals with the intensity of the internal chemical shift standard DSS (4,4-dimethyl-4-silapentane-1-sulfonate). The concentration of the DSS was previously measured by the same method with a solution of *N*-acetyl-tryptophanamide, the concentration of which was determined spectrophotometrically.

2.2.1. PBD–Cdc25C-P. The PBD fragment was mixed with the phosphopeptide in a molar ratio of 1:2. Crystals were obtained at 289 K in 100 m*M* HEPES pH 7.5 and 2 *M* ammonium formate mother liquor. 1 µl of the PBD (7 mg ml^{-1})/phosphopeptide preparation was mixed with 1 µl mother liquor. The drop was equilibrated using 500 µl mother liquor. Crystals appeared in three to four days with dimensions of 10 × 5 × 5 µm (Fig. 1*a*); they were equilibrated in a cryoprotectant buffer composed of the mother liquor plus 15% glycerol and frozen by direct immersion in liquid nitrogen.

2.2.2. PBD–Cdc25C. The peptide was mixed with the PBD fragment in a 2:1 stoichiometric ratio. Crystals were grown by vapour

Table 1
Data-collection statistics for the PBD–Cdc25C-P complex.

Resolution limits (Å)	$\langle I/\sigma(I)\rangle$	Completeness (%)	Multiplicity	Observed reflections	Unique reflections	R_{sym}†
50.0–4.52	9.7	85.9	4.7	2230	1323	0.037
4.52–3.59	13.6	88.9	4.9	2283	1287	0.042
3.59–3.14	12.8	90.0	4.9	2296	1280	0.065
3.14–2.85	9.5	91.0	4.9	2321	1279	0.093
2.85–2.65	6.7	91.9	4.9	2359	1290	0.136
2.65–2.49	5.1	92.8	4.9	2374	1294	0.178
2.49–2.37	4.2	93.2	5.0	2396	1296	0.215
2.37–2.26	3.6	93.1	4.7	2335	1273	0.242
2.26–2.18	2.6	92.6	4.9	2344	1271	0.277
2.18–2.10	2.1	87.8	3.9	2145	1214	0.290
Overall	8.0	90.6	4.8	23083	12807	0.091

† $R_{sym} = \sum_h \sum_i |I_{h,i} - \langle I_h\rangle| / \sum_h \sum_i |I_{h,i}|$.

Table 2
Data-collection statistics for the PBD–Cdc25C complex.

Resolution limits (Å)	$\langle I/\sigma(I)\rangle$	Completeness (%)	Multiplicity	Observed reflections	Unique reflections	R_{sym}†
50.0–9.01	7.7	84.2	2.3	317	128	0.07
9.01–6.37	5.6	91.3	2.5	642	242	0.08
6.37–5.20	6.8	93.3	2.6	913	324	0.08
5.20–4.51	7.0	93.9	2.7	1056	374	0.07
4.51–4.03	7.0	94.7	2.6	1207	425	0.07
4.03–3.68	6.9	95.3	2.6	1362	476	0.09
3.68–3.41	4.3	95.5	2.7	1449	506	0.13
3.41–3.19	3.3	95.9	2.6	1564	552	0.17
3.19–3.00	2.5	96.1	2.6	1631	580	0.22
3.00–2.85	1.9	96.3	2.6	1750	618	0.31
Overall	4.8	96.3	2.6	11891	4225	0.09

† $R_{sym} = \sum_h \sum_i |I_{h,i} - \langle I_h\rangle| / \sum_h \sum_i |I_{h,i}|$.

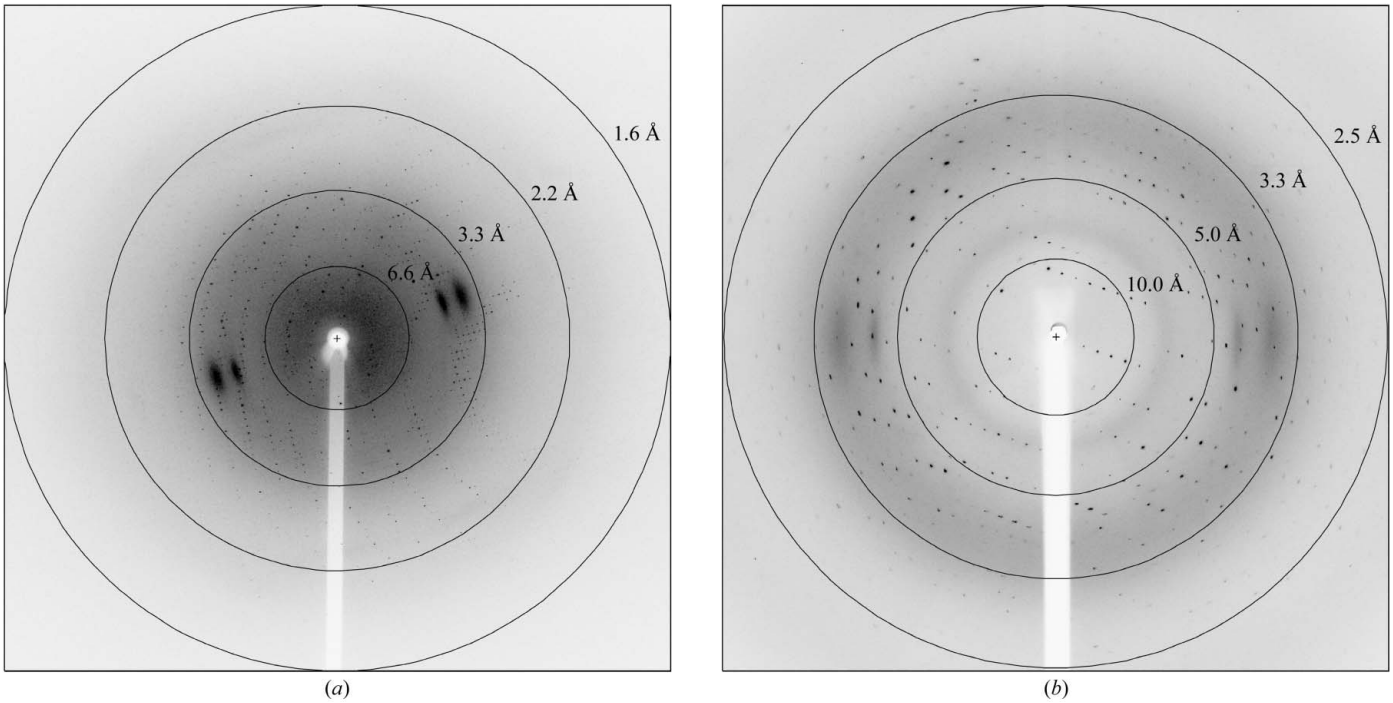
diffusion at 289 K in hanging drops using 100 m*M* HEPES pH 7.5 and 20% PEG 10 000 as mother liquor. 1 µl of the PBD (7 mg ml^{-1})/peptide preparation was mixed with 1 µl mother liquor. The drop was equilibrated using 500 µl mother liquor. Small crystals grew in a week to dimensions of around 5 × 10 × 5 µm (Fig. 1*b*). These crystals were mounted in litholoops (Molecular Dimensions, UK) and cryoprotected by transfer to 15% glycerol prior to flash-cooling in liquid nitrogen.

2.3. Data collection and reduction

All data were collected at cryogenic temperatures using synchrotron radiation at 100 K. The PBD crystals in complex with Ccd25C-P and Cdc25C target peptides were mounted and cryoprotected as previously mentioned. Data sets from PBD–Cdc25C-P and PBD–Cdc25 were collected using synchrotron radiation at the ID14-4 and ID23-1 beamlines at the ESRF (Grenoble, France) and at the PX beamline at the SLS (Villigen, Switzerland). Diffraction data were recorded on ADSC-Q4 or MAR 225 CCD detectors depending on the beamline. The best data sets (see Tables 1 and 2) were collected using $\Delta\varphi = 1^\circ$ and a wavelength of 0.980 and 0.976 Å, respectively (Fig. 2). Processing and scaling were accomplished with *HKL*2000 (Otwinowski & Minor, 1997) or *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1993) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Statistics for the crystallographic data are summarized in Tables 1 and 2.

3. Results and discussion

The recombinant human PBD (residues 367–603) has a molecular weight of 26 kDa. This fragment of Plk1 was expressed as a GST-fusion protein in *E. coli*, with typical yields of 15 mg of pure protein per litre of culture. The fusion protein was subjected to GST-affinity purification and the GST tag was removed using 3C precision protease. The GST was separated from the PBD using a gel-filtration purification step. The purified PBD was concentrated and then used



(*a*) (*b*)

Figure 2
Diffraction patterns obtained from the native crystals using synchrotron radiation. (*a*) PBD–Cdc25C phosphorylated crystals using the PX beamline at the SLS. (*b*) PBD–Cdc25C unphosphorylated crystals using ID23-1 at the ESRF.

for cocrystallization assays. Although small crystals were relatively easy to obtain using the nanodrop crystallization robot, it took several rounds of refinement of the crystallization conditions in order to reproduce the crystals in hanging drops with a 2 µl volume. After cocrystallization with different target peptides, their presence in the crystals was checked by mass spectrometry (data not shown). The PBD in complex with Cdc25C-P and Cdc25-C target peptide crystals diffracted to 2.1 and 2.85 Å resolution, respectively, and several data sets were obtained. The PBD–Cdc25C-P crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 38.23$, $b = 67.35$, $c = 88.25$ Å, $\alpha = \gamma = \beta = 90^\circ$. The Matthews coefficient and the self-rotation function (data not shown) suggested the presence of one protein molecule per asymmetric unit ($V_M = 2.2$ Å^3 Da^{-1}) and a solvent content of 43%. The diffraction data collected has 90.3% completeness, a multiplicity of 4.8 and an overall $I/\sigma(I)$ of 8.0. Crystals of the PBD in complex with Cdc25C target peptide belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 40.18$, $b = 49.17$, $c = 56.23$ Å, $\alpha = \gamma = 90$, $\beta = 109.48^\circ$, and the self-rotation function (data not shown) suggested the presence of one molecule per asymmetric unit with a Matthews coefficient V_M of 2.0 Å^3 Da^{-1} (Matthews, 1968) and a solvent content of 39%. The data has 96.3% completeness, a multiplicity of 2.6 and an overall $I/\sigma(I)$ of 4.6.

Probably owing to the crystal size, data sets could only be collected at low resolution using an in-home source at 110 K. Therefore, a cryobuffer was selected and initial diffraction tests were performed using a synchrotron-radiation source. Although small crystals were easily obtained, several rounds of refinement were needed in order to obtain crystals with a reasonable size for data collection. Crystallization reproducibility was purification-dependent and some batches did not yield crystals or were of very poor quality. Finally, several native data sets were collected at 100 K on beamlines ID14-4 and ID23-1 at the ESRF and at the PX beamline at the SLS. Using synchrotron radiation on these undulator-equipped beamlines, the crystals diffracted to 2.10 and 2.85 Å. The statistics for both data sets are given in Tables 1 and 2. The structures of both complexes have been solved by molecular replacement using the coordinates from PDB entry 1umw (Elia, Rellos *et al.*, 2003). In both structures a clear extra density could be observed corresponding to the peptide. These are the first crystals of the Polo-box domain (PBD) in complex with a physiological substrate both in its unphosphorylated and phosphorylated state. The structures reveal that sequence recognition is an important issue despite phosphorylation and suggest a discrimination mechanism between the different phosphorylation states based on the loop which connects the Polo boxes (García-Álvarez *et al.*, unpublished data). We believe that these studies will help to decipher in detail the basis of the molecular recognition between Plk1 and its substrates, a central theme in mitosis.

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