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Purification, crystallization and preliminary X-ray diffraction of a proteolytic fragment of PDK1 containing the pleckstrin homology domain

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3-Phosphoinositide-dependent protein kinase-1 (PDK1) is a Ser/Thr kinase with an essential role in insulin and growth-factor signalling. PDK1 activity towards protein kinase B (PKB) is partially regulated by its pleckstrin homology (PH) domain, which preferentially binds to 3-phosphoinositides. However, the precise molecular mechanism of this regulation is not well understood. Here, the cloning, purification and crystallization of a 150-amino-acid C-terminal region of PDK1 containing the PH domain is reported. A crystal of the PDK1 PH domain grown in the presence of inositol 1,3,4,5-tetrakisphosphate and derivatized with AuCN diffracted to 1.5 Å at a synchrotron source. Diffraction data collected near the Au edge resulted in an anomalous Patterson map with a 30σ peak.

1. Introduction

PDK1 is a member of the AGC family of protein kinases with a central role in phosphatidylinositol 3-kinase signalling (Alessi, 2001). It activates a number of other AGC family protein kinases, a key member of which is protein kinase B (PKB, also known as Akt; Brazil & Hemmings, 2001). PKB and PDK1 co-localize at the plasma membrane upon production of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] (Vanhaesebroeck & Alessi, 2000) by binding this lipid second-messenger molecule through their PH domains. PDK1 subsequently activates PKB by phosphorylating its T-loop at Thr308 (PKBα) (Scheid & Woodgett, 2001). For PKB, interaction of the PH domain with its ligand has been shown to induce a conformational change, possibly leading to the presentation of the PKB T-loop for phosphorylation by PDK1 (Milburn *et al.*, 2003; Calleja *et al.*, 2003). However, the regulation of PDK1 by its C-terminal PH domain is less well understood, in particular in terms of its ligand specificity. Previous studies (Currie *et al.*, 1999) have shown that PDK1 interacts with PtdIns(3,4)P_2, PtdIns(3,4,5)P_3 and also, to a lower extent, with PtdIns(4,5)P_2. In terms of sequence homology, the PDK1 PH domain is not detected by sequence searches with a PH-domain sequence profile. Here, we present a proteolytic approach to obtain a crystallizable construct for the PH domain of PDK1 and describe subsequent protein purification and crystallization. Furthermore, we describe initial X-ray diffraction studies on a crystal grown in the presence of inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] and derivatized with AuCN in order to evaluate the potential anomalous signal for structure determination.

2. Obtaining construct boundaries

Constructs for the PH domain of PDK1 were initially designed based on sequence alignments with sequences of the previously crystallized PH domains of TAPP1 (Thomas *et al.*, 2001), DAPP1 (Ferguson *et al.*, 2000), PKBα (Thomas *et al.*, 2002) and PEPP1 (Milburn *et al.*, 2004). Four 90–120-amino-acid constructs were cloned from human cDNA, sequence-validated and transferred to the bacterial expression vector pGEX-6P (Amersham Biosciences), resulting in inducible expression as cleavable GST-fusion proteins. All four constructs showed protein-stability problems after cleavage of the GST tag, resulting in insufficient amounts for crystallization trials. To obtain the construct boundaries for a soluble PDK1 PH domain, PDK1 (residues 51–556, purified from baculovirus-infected SF21 insect cells as described previously; Biondi *et al.*, 2002) was used in a limited proteolysis approach. 20 µg of PDK1 was cleaved with 0.2 µg trypsin in 100 m*M* NaCl, 50 m*M* Tris pH 8.0 at 303 K. Aliquots were taken every 1 min over 20 min, supplemented with 10 m*M* benzamidine, and subjected to SDS–PAGE analysis (Fig. 1). Tryptic cleavage of PDK1 resulted in four distinct bands with approximate molecular weights of 40, 37, 21 and 17 kDa (Fig. 1), which were analysed by N-terminal Edman sequencing. The fragments at 40 and 37 kDa showed the N-terminal sequence of the full-length protein and the

21 kDa fragment started in the T-loop of the kinase domain cleaved after residue Arg238. However, the 17 kDa band consisted of a fragment starting at Ser408 and therefore contained the PH domain. MALDI–TOF analysis of the band showed a single peak at 17 566 Da corresponding to the fragment of PDK1 comprising residues 408–556 (theoretical MW = 17 608 Da).

3. Cloning, expression and purification

A construct for PDK1 408–556 was generated from human cDNA using the primers 5′-AGATCTAACATAGAGCAGTACATTCACGATCTG-3′ (forward) and 5′-TCACTGCACAGCGGCGTCCGGGTGGCTC-3′ (reverse). The amplified PCR product was ligated into pCR TOPO vector (Invitrogen), sequenced and subcloned as a *Bgl*II–*Eco*RI fragment into the *Bam*HI–*Eco*RII site of pGEX-6P1. GST-fusion protein was expressed using *Escherichia coli* BL21(DE3) cells. Cells were grown in 4 l Luria–Bertani broth medium containing 50 µg ml^{-1} carbenicillin at 310 K to an OD_{600} of 0.9, induced with 250 µ*M* isopropyl-β-D-thiogalactopyranoside and grown for 16 h at room temperature. The cells were harvested by centrifugation at 3500*g* for 20 min and then lysed by resuspension in 200 ml lysis buffer (150 m*M* sodium chloride, 270 m*M* sucrose, 50 m*M* Tris pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* sodium orthovanadate, 10 m*M* sodium glycerophosphate, 50 m*M* sodium fluoride, 10 m*M* β-mercaptoethanol, 1 m*M* benzamidine, 1 m*M* phenylmethanesulfonyl fluoride) containing additional DNAse I (0.1 mg ml^{-1}) and lysozyme (1 mg ml^{-1}). After incubation on ice for 30 min and brief sonication, the lysate was cleared by centrifugation and incubated for 1 h at 277 K with 10 ml glutathione Sepharose beads (Amersham Biosciences). The resin was washed with 200 ml lysis buffer, 200 ml buffer *A* (500 m*M* NaCl, 50 m*M* Tris pH 7.0, 2.5 m*M* DTT) and 2 l buffer *B* (300 m*M* NaCl, 50 m*M* Tris pH 7.0, 2.5 m*M* DTT). Cleavage was performed with 200 µg GST-tagged PreScission protease for 8 h at 277 K. The resulting supernatant containing cleaved PDK1 PH domain was concentrated to 5 ml and loaded onto a 16/60 Superdex 200 gel-filtration column equilibrated in buffer *B* on an AKTA Explorer system (Amersham Biosciences). Pure PDK1 PH domain was then concentrated from appropiate fractions to 7 mg ml^{-1} using a VivaSpin 2 ml concentrator (10 000 Da molecular-weight cutoff, Viva-Science).

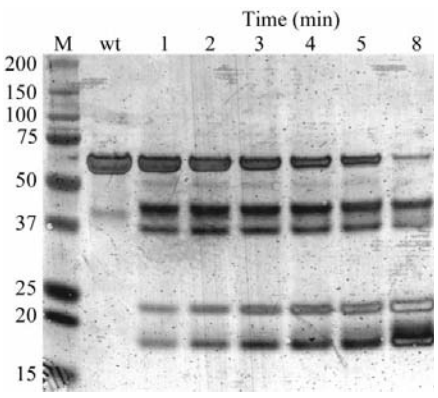

Figure 1
Time course (1–8 min) of limited proteolysis on PDK1 (wt), resolved by SDS–PAGE. The lowermost band at 17 kDa contains a proteolytically stable fragment comprising the PDK1 PH domain. Lane M, molecular-weight markers (kDa).

4. Crystallization

Purified PDK1 PH domain was mixed with a 25-fold molar excess of Ins(1,3,4,5)P_4 (Cell Signals, Columbus OH, USA) and used in crystallization experiments with commercially available sparse-matrix screens (Hampton Research, Decode Genetics). 0.75 µl protein solution was mixed with 0.75 µl reservoir solution in a sitting-drop vapour-diffusion setup and incubated at 293 K. Clusters of thin plates formed overnight in a number of conditions. Crystals grown from 0.08 *M* magnesium acetate, 0.05 *M* sodium cacodylate pH 6.5, 30%(*w*/*v*) PEG 4000 reached dimensions of up to 0.4 × 0.4 × 0.1 mm (Fig. 2). The crystals were soaked for 10 s in mother liquor containing 5% glycerol prior to flash-cooling in a nitrogen Cryostream. For heavy-atom soaking experiments, a 10 m*M* solution of AuCN was prepared in the mother liquor and individual crystals were soaked for 4 h at 293 K. Prior to cooling, the crystals were soaked in cryoprotectant containing no heavy atoms.

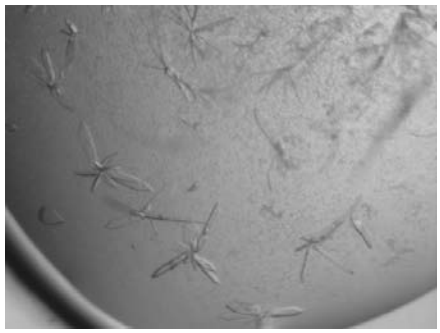

Figure 2
Crystals of the PDK1 PH domain–Ins(1,3,4,5)P_4 complex.

Table 1
Data statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.861
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 35.40, b = 58.92, c = 36.58, β = 101.48
Resolution (Å)	25.0–1.50 (1.58–1.50)
Observed reflections	85433
Unique reflections	23341 (2363)
Redundancy	3.7 (3.3)
Completeness	98.6 (99.5)
R_{merge}	0.076 (0.439)
Average $I/\sigma(I)$	17.7 (3.2)

5. Data collection and Patterson map calculation

Diffraction data were collected at the European Synchrotron Radiation Facility (Grenoble, France) on beamline BM14 at 100 K. Processing and scaling of the data was performed with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997) and further analysis was performed using programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) (Table 1). The crystals were monoclinic (space group $P2_1$), with unit-cell parameters a = 35.40, b = 58.92, c = 36.58 Å, β = 101.48°. Matthews coefficient calculation suggested one molecule per asymmetric unit (V_M = 2.1 Å^3 Da^{-1}, with 41% solvent content). A fluorescence scan indicated the presence of bound Au from the soaking experiment. A data set was collected to 1.50 Å resolution at the Au L_I edge (λ = 0.861 Å). Analysis of the Harker section at v = 0.5 in the anomalous difference Patterson map revealed one 30σ peak (Fig. 3).

6. Conclusions

We have designed and expressed a soluble fragment corresponding to the PDK1 PH domain *via* a strategy of limited proteolysis. A suitable derivative has been identified. Structure solution is under way and may reveal the structure of the PDK1 PH

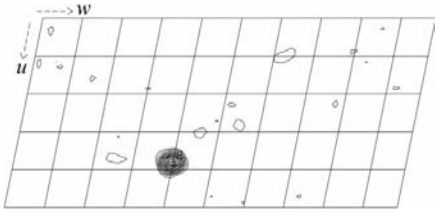

Figure 3
Anomalous difference Patterson map of a Au-derivatized crystal of the PDK1 PH domain–Ins(1,3,4,5)P_4 complex. The Harker section at v = 0.5 is shown. Peaks are contoured in 2.0σ intervals starting at 2.0σ. The peak height of the major peak is 30σ.

domain. This would represent the first structural example of a less specific 3-phosphoinositide-binding domain and may provide further insight into the regulation of PDK1 by its PH domain.

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