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Expression, purification, crystallization and preliminary crystallographic analysis of human Pim-1 kinase

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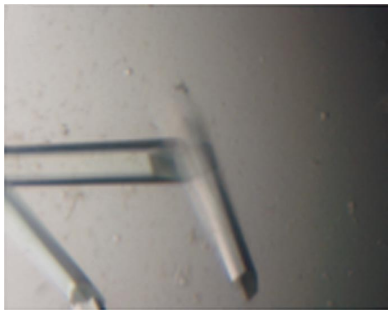

Pim kinases, including Pim-1, Pim-2 and Pim-3, belong to a distinctive serine/threonine protein-kinase family. They are involved in cytokine-induced signal transduction and the development of lymphoid malignancies. Their kinase domains are highly homologous to one another, but share low sequence identity to other kinases. Specifically, there are two proline residues in the conserved hinge-region sequence ERP*X*P*X* separated by a residue that is non-conserved among Pim kinases. Full-length human Pim-1 kinase (1–313) was cloned and expressed in *Escherichia coli* as a GST-fusion protein and truncated to Pim-1 (14–313) by thrombin digestion during purification. The Pim-1 (14–313) protein was purified to high homogeneity and monodispersity. This protein preparation yielded small crystals in the initial screening and large crystals after optimization. The large crystals of apo Pim-1 enzyme diffracted to 2.1 Å resolution and belong to space group $P6_5$, with unit-cell parameters $a = b = 95.9$, $c = 80.0$ Å, $\beta = 120^\circ$ and one molecule per asymmetric unit.

1. Introduction

Pim-1 is a serine/threonine protein kinase (Saris *et al.*, 1991) with oncogenic function. It was originally identified as a common integration site in Moloney murine leukemia virus (MMLV) induced T-cell lymphomas (Cuypers *et al.*, 1984). Overexpression of Pim-1 in mice resulted in susceptiblity to lymphomagenesis, which is likely to occur through a synergistic effect between Pim-1 and c-myc (van Lohuizen *et al.*, 1989). In humans, abnormal Pim-1 expression was detected in patients with lymphoma (Yoshida *et al.*, 1999) and leukemia (Amson *et al.*, 1989). Pim-2 was also identified as a common insertion site for MMLV and possessed similar oncogenic functions to Pim-1 (Breuer *et al.*, 1989; van der Lugt *et al.*, 1995). Pim-3 has not been as well studied as Pim-1 and Pim-2, but is believed to have overlapping functions with Pim-1 and Pim-2.

Pim-1 plays a role in cell-growth control and survival (Wang *et al.*, 2001). Expression of Pim-1 is induced by a variety of growth factors, cytokines and mitogens, suggesting that Pim-1 may be involved in signal transduction initiated from these factors. Recently, mice deficient in all Pim kinases were shown to have reduced body size throughout their life span and the hematopoietic cell populations from these mice were defective in response to a subset of growth-factor stimulation (Mikkers *et al.*, 2004). In hematopoietic cells, overexpression of Pim-1 enhances cellular survival, reportedly by protecting these cells from apoptosis induced by cytokine withdrawal (Lilly *et al.*, 1999; Shirogane *et al.*, 1999), glucocorticoids (Moroy *et al.*, 1993) and genotoxic stress (Pircher *et al.*, 2000).

At the sequence level, Pim-2 and Pim-3 have 61 and 71% identity to Pim-1 (Allen & Berns, 1996), suggesting that Pim kinases have similar three-dimensional structures. However, they are less than 30% identical in sequence to other known protein kinases. The Pim-family kinases also share a consensus hinge-region sequence ERP*X*P*X*, which is quite unique because of the two proline residues. This unique sequence may lead to a hinge-region architecture that is distinct from other kinase hinge regions (Knighton *et al.*, 1991; Johnson *et al.*, 1996). Therefore, structure elucidation of Pim-1 kinase should provide structural insights into Pim-1 as well as Pim-2 and Pim-3 kinases and the precise hinge-region conformation of Pim-1 kinase should greatly benefit structure-based drug-design efforts

targeting any of the Pim kinases. Here, we report the cloning, expression, crystallization and preliminary crystallographic analysis of human Pim-1 kinase.

2. Material and methods

2.1. Cloning and expression

The full-length Pim-1 cDNA was amplified by PCR using IMAGE clone 4591723 (GenBank entry BC020224.1) as a template. The forward and reverse primers are 5′-GGG **GAA TTC** ATG CTC TTG TCC AAA ATC-3′ and 5′-GGG G**AA GCT T**CT ATT TGC TGG GCC CCG GCG AC-3′, which contain the restriction sites for *Eco*RI and *Hin*dIII, respectively (in bold). The PCR-amplified DNA fragment was purified, digested with *Eco*RI and *Hin*dIII and ligated into pET41a vector (Novagen). The ligated vector carries a GST tag, a His tag and an S tag that are in tandem and N-terminal to the full-length Pim-1 gene, with an engineered thrombin-cleavage site between the last two tags. The cloned Pim-1 gene was verified by sequencing.

The pET41a-Pim1 construct was expressed in *Escherichia coli* strain BL21 Star (DE3) pLysS (Invitrogen) in LB media. A 0.5 ml aliquot of a frozen glycerol stock was used to inoculate four 500 ml LB cultures containing 50 mg l^{-1} kanamycin and 34 mg l^{-1} chloramphenicol. These inoculation cultures were grown overnight at 310 K. The cultures were then used to inoculate a 15 l stirred tank fermentor at 310 K. When the OD_{600} of the 15 l culture reached 1.0, the temperature was reduced to 298 K and the culture was induced with 0.5 m*M* IPTG. The culture was harvested after 3 h induction. A typical fermentation yielded about 55 g of cell mass. The cell mass was frozen in liquid nitrogen and stored at 193 K until use.

2.2. Purification

Briefly, Pim-1 kinase was purified using GST-affinity chromatography, 'on-resin' thrombin cleavage and anion-exchange and size-exclusion chromatography (SEC) at 277 K. The protein concentrations were calculated from the absorption at 280 n*M* wavelength (A_{280}) using a calculated molar extinction coefficient value of 49 510 (1 mg ml^{-1} = 1.5 unit of A_{280}). About 60 g cell paste was resuspended in 120 ml lysis buffer containing 20 m*M* Tris pH 8.0, 500 m*M* NaCl, 2 m*M* $CaCl_2$, 5 m*M* DTT, 5 µg ml^{-1} leupeptin, 5 µg ml^{-1} pepstatin, 1 m*M* *p*-toluenesulfonyl chloride (PMSCl) and 10 units ml^{-1} DNAse. The suspension was then passed through a cell disruptor three times followed by centrifugation at 48 000*g* for 15 min. The supernatant was collected and immediately loaded at 4 ml min^{-1} onto a pre-washed XK50 column (Amersham) packed with 50 ml Glutathione-Sepharose 4B resin (GST-resin, Amersham). The loaded resin was immediately washed first with 400 ml of a buffer containing 20 m*M* Tris pH 8.0, 500 m*M* NaCl, 2 m*M* $CaCl_2$, 5 m*M* DTT at 4 ml min^{-1} and 1 ml min^{-1} (for the last 100 ml), then with 120 ml QA buffer (20 m*M* Tris pH 8.0, 50 m*M* NaCl, 5 m*M* $CaCl_2$, 5 m*M* DTT). The GST-resin bound with Pim-1 fusion protein was incubated overnight with 5000 units of thrombin (Sigma-Aldrich) dissolved in 10 ml QA buffer. The digested Pim-1 was eluted from the GST-resin using QA buffer and peak fractions were loaded directly onto a pre-equilibrated (QA buffer) 8 ml Mono-Q column (Amersham) for anion-exchange chromatography. The Pim-1 protein eluted between 150 and 250 m*M* NaCl using a 0–1 *M* gradient in ten column volumes. Most fractions contained more than four major bands of protein on SDS–PAGE, three of which are close to the size of Pim-1 protein. These fractions were pooled (50 ml) and digested again in solution using 10 000 units of thrombin overnight. The re-digested sample was diluted and subjected to anion-exchange chromatography as described above. The peak fractions were pooled (40 ml, 1 mg ml^{-1}) and concentrated to about 4.5 mg ml^{-1} for SEC. About 3 ml of the concentrated sample was loaded onto a pre-equilibrated Superdex-75 (preparative, 120 ml resin) column (Amersham) followed by elution using S buffer (20 m*M* Tris pH 8.0, 200 m*M* NaCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 5 m*M* DTT). The purified Pim-1 sample was characterized using SDS–PAGE, dynamic light scattering using DynaPro-800 (Proterion), N-terminal sequencing and mass spectrometry. The peak fractions were pooled, concentrated to 4 mg ml^{-1}, aliquoted (400 µl), flash-frozen using liquid nitrogen and stored at 193 K.

2.3. Crystallization

The purified aliquot of Pim-1 protein was thawed out and applied onto a pre-equilibrated Superdex-75 column (analytical, 30 ml resin) followed by elution using S buffer. The major peak fractions were pooled and concentrated to about 4 mg ml^{-1} for crystallization trials both as apoprotein and as an AMP-PNP (1 mg ml^{-1} final concentration) complex against 96 sparse-matrix conditions (Hampton, HR2-132) and an in-house grid screen. The in-house grid was comprised of 48 conditions permuted from PEG 1500 (18, 24, 30, 36%) and PEG 4000 (12, 16, 20, 24%) at six different pH values (ADA for pH 6.0, 6.5; Tris for pH 7.0, 7.5, 8.0, 8.5). The screening was conducted using hanging drops consisting of 0.5 µl each of protein and well solutions. Larger crystals were obtained by optimization using hanging drops with 1.5 µl each of protein and well solutions. Crystals were grown at room temperature and the final crystallization solution contained 30% PEG 1500, 0.1 *M* Tris pH 7.5 and 0.3 *M* NaCl.

2.4. Preliminary crystallographic analysis

Examination of crystal diffraction and data collection were conducted using an R-AXIS IV^{++} imaging system mounted on a RU300 rotating-anode X-ray generator (Rigaku/MSC) at cryogenic temperature. The Pim-1 crystals were cryoprotected using well solution with the addition of 5% glycerol. The diffraction data were processed using the *HKL*2000 package (Otwinowski & Minor, 1997).

3. Results and discussion

Initial attempts to express the GST-tagged Pim-1 gene using the pDEST15 gateway vector were unsuccessful and the data generated from these trials suggested that the construct was unstable (data not shown). The Pim-1 gene was then subcloned into the pET41a vector, which contains a constitutively expressed Lac^{IQ} protein that decreases read-through expression during the growth phase. Expression of the GST-Pim1 construct from this vector was successful as described and was highly reproducible. In addition, the purification protocol for Pim-1 was optimized by eliminating the buffer-exchange step normally required prior to ion-exchange chromatography. Trial experiments showed that both thrombin cleavage of Pim-1 and the binding of digested Pim-1 to the anion-exchange (Mono-Q) column could be carried out in low-salt QA buffer. Elimination of this step improved the overall efficiency of the protocol because buffer exchange is usually time-consuming and achieves no purification. The divalent anions Mg^{2+} and Ca^{2+} were included in the SEC and the final protein buffer as they were found to be beneficial in stabilizing the Pim-2 protein (data not shown). Thrombin digestion in one step using 15 000 units of enzyme was also found to be as effective (data not shown) as two-step digestion as described above.

The encoded GST-fusion protein of human Pim-1 kinase contains one engineered and two endogenous thrombin-cleavage sites at

LVPR·GST (−48·−47), GYR·GSEF (−5·−4) and HLR·AAP (13·14) between R·G(A). Cleavage at these sites would produce three different proteins: Pim-1 (−47–313), Pim-1 (−4–313) and Pim-1 (14–313), with expected molecular weights of about 40, 36 and 34 kDa, respectively. The initial digestion using 5000 units of thrombin produced a mixture of more than four protein species, three of which co-eluted on anion-exchange chromatography with molecular weights around 35 kDa (Fig. 1*a*) and possibly corresponded to Pim-1 species from different cleavage sites. Further digestion of the mixture using 10 000 units of thrombin increased the relative abundance of the 34 kDa protein, with the appearance of some extra thrombin-related protein bands (Fig. 1*b*). The extra protein bands after digestion were removed using anion-exchange chromatography (Fig. 1*c*). This sample was further purified by preparative SEC and produced a major peak with a retention volume corresponding to a monomeric Pim-1 protein. The major peak fractions were pooled and concentrated to about 4 mg ml^{-1}, and yielded a total of about 24 mg Pim-1 protein.

The purified protein was found to be highly homogeneous on SDS–PAGE (Fig. 1*d*). The results from dynamic light scattering (Ferre-D'Amare & Burley, 1994, 1997) show that a fresh Pim-1 (14–313) sample is monodisperse ($\Delta R/R$ < 15%, baseline < 1.005) at concentrations up to 4 mg ml^{-1}, with a molecular weight around 40 kDa (Table 1); the results also suggest the presence of monomeric Pim-1 in solution. N-terminal sequencing shows there is only one protein species detectable in the purified Pim-1 sample, with a sequence AAPWNDJHATMLAPG, exactly matching that of Pim-1 (14–313). Mass spectrometry shows a 70% parent-ion peak of 34 207 Da, an exact match with the calculated peak of 34 206 Da for Pim-1 (14–313), and a 30% peak of 34 346 Da, possibly being a DTT adduct of Pim-1 (14–313). These results establish that human Pim-1 (14–313) kinase was obtained with high purity and monodispersity.

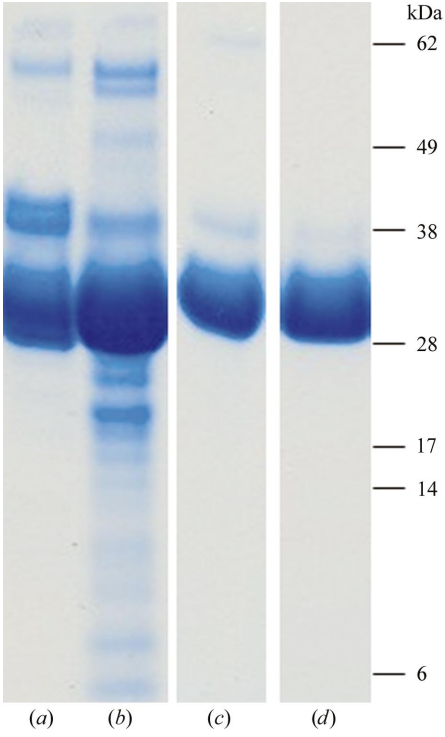

Figure 1
Human Pim-1 kinase samples obtained at different stages of purification. These results were obtained from three independent SDS–PAGE experiments. (*a*) Raw Pim-1 protein sample obtained by digesting the GST-fusion protein using 5000 units of thrombin followed by anion-exchange chromatography. (*b*) Pim-1 protein obtained after a second digestion with an additional 10 000 units of thrombin. (*c*) Pim-1 protein purified using anion-exchange chromatography from twice-digested GST-fusion Pim-1 protein. (*d*) Pure Pim-1 protein obtained using preparative SEC.

Table 1
Dynamic light-scattering analysis of Pim-1 (14–313).

Samples (mg ml^{-1})	$\Delta R/R$ (%)	$\Delta R/R$ (nm)	MW (kDa)	Count rate	Baseline	SOS
0.7	13.6	0.4/2.94	39.6	24000	1.001	8.14
4.1	12.6	0.4/3.17	47.7	124000	1.001	0.64
0.7†	30.6	1.0/3.26	51.3	24700	1.011	8.65
4.1†	20.7	0.7/3.37	55.3	127000	1.007	1.91

† The sample was frozen/thawed once.

Pim-1 (14–313) protein becomes polydisperse ($\Delta R/R$ > 15%, baseline >1.005) after being subjected to a single freeze–thaw cycle (Table 1). Because systematic studies show that monodisperse protein samples are more readily crystallized than polydisperse samples (Ferre-D'Amare & Burley, 1997), the frozen aliquots of the protein were repurified through an analytical SEC column. The major peak was pooled and concentrated to about 4 mg ml^{-1} and was determined to be monodisperse again. All samples used for crystallization trials were repurified using SEC. Small crystals were found in 17 of the 96 sparse-matrix conditions and 37 of the 48 in-house grid conditions with the presumed AMP-PNP complex, but in only two sparse-matrix conditions for the apoenzyme. Crystals obtained from samples containing AMP-PNP could not be made larger and were not used for structure studies; however, they were valuable as seeds for apoenzyme crystallization in conditions originally found in AMP-PNP-containing trials. Since the morphology of the small crystals from the in-house grid conditions looked promising (Fig. 2*a*), these conditions were used to start optimizing apo Pim crystallization coupled with seeding. Using modified grid solutions containing 18% PEG 1500, 0.1 *M* Tris pH 7.5 and 0.2 *M* $MgCl_2$ or 30% PEG 1500, 0.1 *M* Tris pH 7.5 and 0.3 *M* NaCl (final), the apo protein yielded crystals with widths of 20 µm (Fig. 2*b*) or 40 µm (Fig. 2*c*) that diffracted to 3.3 or 2.5 Å resolution, respectively.

Pim-1 (14–313) protein sample was still potentially contaminated by a trace amount of Pim-1 (−4–313) arising from incomplete

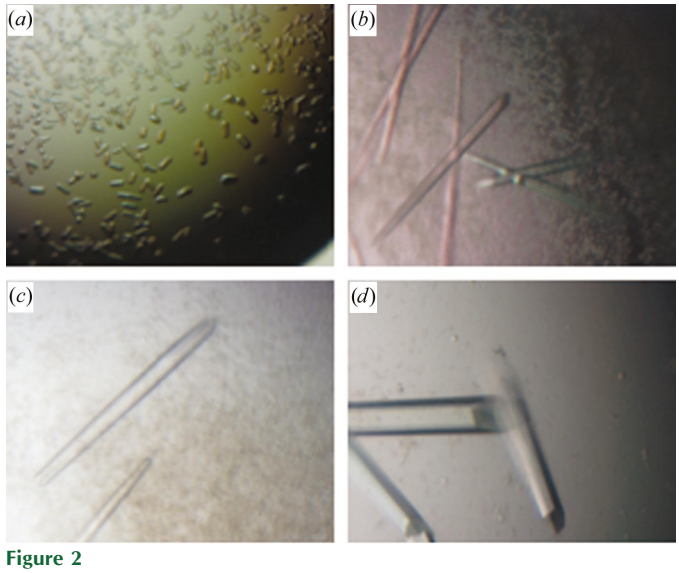

Figure 2
A panel of Pim-1 (14–313) kinase crystals grown under different conditions with the same magnification. (*a*) Small crystals of Pim-1–AMP-PNP sample. (*b*) Crystals of apo Pim-1 enzyme of 20 µm width. (*c*) Crystals of apo Pim-1 enzyme of 40 µm width. (*d*) Crystals of apo Pim-1 enzyme of 100 µm width.

Table 2
Statistics of crystallographic data.

Values in parentheses are for the last resolution shell.

Crystal width (µm)	100
Space group	$P6_5$
Unit-cell parameters	
a (Å)	95.9
c (Å)	80.0
β	120
Molecules per AU	1
Resolution (Å)	50–2.1 (2.14–2.10)
Completeness (%)	99.7 (99.6)
$\langle I/\sigma(I)\rangle$	17.8 (3.09)
$R_{\rm merge}$†	0.106 (0.511)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I - \langle I\rangle| / \sum_{hkl}\sum_i I$.

digestion beyond the detection limits of the characterization techniques. We hypothesized that even minute amounts of Pim-1 (−4–313) could potentially poison the crystal lattice of Pim-1 (14–313) and compromise crystal growth. Following this hypothesis, we repurified the aliquoted pure Pim-1 sample twice using analytical SEC to further reduce the contamination. The twice repurified Pim-1 sample yielded crystals of approximate dimensions 100 × 100 × 1000 µm (Fig. 2*d*) using the final crystallization condition that diffracted to 2.1 Å resolution. Seeding was necessary to grow large Pim-1 crystals (20, 40 or 100 µm) because the number of crystals in a drop was roughly correlated to the volume of seeding solution and no crystals were found if the drop was not seeded.

The Pim-1 crystals of various sizes all belong to the same space group with very similar unit-cell parameters. The 20 µm crystals diffracted only weakly to 3.3 Å resolution, probably owing to both the smaller crystal size and the slightly longer a axis (data not shown). X-ray diffraction data were collected for the various sizes of Pim-1 crystals and the statistics of the 2.1 Å data are summarized in Table 2. The data indicate a hexagonal space group, either $P6_5$ or $P6_1$, with unit-cell parameters $a = b = 95.9$, $c = 80.0$ Å, $\beta = 120^\circ$. Assuming the presence of one molecule in the asymmetric unit, the Matthews volume (Matthews, 1968) was calculated to be 3.09 Å^3 Da^{-1}. The structure has been determined using molecular replacement in space group $P6_5$ (Qian *et al.*, 2005).

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