Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Expression, crystallization and preliminary X-ray diffraction analysis of human paired Ig-like type 2 receptor *α* (PILR*α*)

Shigekazu Tabata,[a] Kimiko Kuroki,[a] Nobuo Maita,[a] Jing Wang,[b] Ikuo Shiratori,[b] Hisashi Arase,[b,c,d] Daisuke Kohda[a] and Katsumi Maenaka[a]*

[a]Division of Structural Biology, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan, [b]Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan, [c]WPI Immunology Frontier Research Center, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan, and [d]SORST, Japan Science and Technology Agency, Saitama 332-0012, Japan

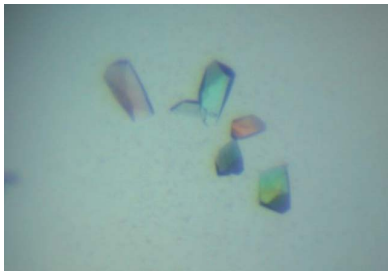
Correspondence e-mail: kmaenaka@bioreg.kyushu-u.ac.jp

Received 17 October 2007
Accepted 4 December 2007

Human paired immunoglobulin-like (Ig-like) type 2 receptor *α* (PILR*α*) is a type I membrane protein that is mainly expressed in immune-related cells such as monocytes, granulocytes and dendritic cells. PILR*α* can suppress the functions of such immune cells because it has the immunoreceptor tyrosine-based inhibitory motif (ITIM) in the intracellular region, which recruits the phosphatase Src homology-2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP-2) to inhibit phophorylations induced by activation signals. The extracellular region of human PILR*α* comprises one immunoglobulin superfamily V-set domain and a stalk region. The V-set domain (residues 13–131) of human PILR*α* was overexpressed in *Escherichia coli* as inclusion bodies, refolded by rapid dilution and purified. The PILR*α* protein was successfully crystallized at 293 K using the sitting-drop vapour-diffusion method. The crystals diffracted to 1.3 Å resolution at SPring-8 BL41XU; they belong to space group $P2_12_12_1$, with unit-cell parameters a = 40.4, b = 45.0, c = 56.9 Å, and contain one molecule per asymmetric unit.

1. Introduction

Immune function is regulated by the balance of activating and inhibitory signals mediated by immune cell-surface receptors (Lanier, 2001; Smith *et al.*, 2001; Moretta *et al.*, 2001; Diefenbach & Raulet, 2003). Among these receptors, there are families that harbour extracellular regions that have a high similarity in amino-acid sequence but have different intracellular domains responsible for activation and inhibitory signalling. Therefore, activating and inhibitory members of these families can generally recognize the same or very similar ligands with slightly different specificities, presumably resulting in fine-tuning of immune regulation. Since they are involved in both the activation and inhibition of immune function, they are generally referred to as paired receptor families. Paired immunoglobulin-like (Ig-like) type 2 receptors (PILRs) are one of the paired receptor families and consist of activating (PILR*β*) and inhibitory (PILR*α*) forms (Fournier *et al.*, 2000; Mousseau *et al.*, 2000; Shiratori *et al.*, 2004). Primary structural analysis indicated that the PILRs have an extracellular region comprising a V-set Ig-like domain and a stalk region, a single spanning transmembrane region and an intracellular region. The amino-acid sequence identity between the extracellular domains of human PILR*α* and PILR*β* is 83%. Human PILR*α* is an inhibitory receptor that suppresses phosphorylations induced by activation signals as it contains the immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits the phosphatase Src homology-2 (SH2) domain-containing protein tyrosine phosphatase 2 (SHP-2) to dephosphorylate activation-induced phosphorylated substrates. The ITIM phosphorylation induced by an anti-PILR monoclonal antibody inhibits calcium mobilization (Fournier *et al.*, 2000). Mousseau and coworkers showed that PILR*α* can also bind to SHP-1 upon tyrosine phosphorylation of the ITIM (Mousseau *et al.*, 2000). On the other hand, our previous cellular-based work demonstrated that mouse PILR*β* can associate with the DAP12 signalling molecule, which has the immunoreceptor tyrosine-based activation motif (ITAM) and can thus activate the functions of natural killer and dendritic cells (Shiratori *et al.*, 2004). Although the physiological



ligand of mouse PILRα and PILRβ was identified as CD99 (Shiratori *et al.*, 2004), the human PILR ligand has not yet been found. However, human PILRα can cross-react with mouse CD99 (Wang *et al.*, unpublished observation), suggesting that human and mouse forms of PILRα have similar ligand-recognition mechanisms. To date, there are no structural data available for any of the PILRs. We sought to determine the three-dimensional structure of PILRα in order to understand the molecular mechanism of its ligand recognition. Here, we report the expression, purification and crystallization of the extracellular V-set domain of human PILRα (residues 13–131).

2. Experimental methods and results

2.1. Expression-vector construction and protein expression

The DNA encoding the human PILRα V-set domain (residues 13–131, designated PILRα) was amplified by the polymerase chain reaction (PCR) using the full-length cDNA of human PILRα as a template (AF16080): 5′-GGA ATT CCA TAT GCT TTA TGG GGT CAC TCA ACC AAA AC-3′ as the forward primer and 5′-CCA AGC TTA CTA GGT GAT GGA GAG TTT GGT CCC-3′ as the reverse primer. The resultant PCR fragment including the V-set domain with additional N-terminal methionine was digested with the *Nde*I and *Hin*dIII restriction enzymes and was cloned into the pGMT7 vector (Reid *et al.*, 1996), which contains the T7 promoter for overexpression in *Escherichia coli*.

The plasmid was transformed into *E. coli* Rosetta (DE3) competent cells (Novagen) and a single colony was inoculated into 5 ml 2YT medium at 310 K and cultured overnight. The overnight culture was then transferred into a flask containing 1 l 2YT medium with 100 mg l^{-1} ampicillin (Nacalai Tesque, Japan). When the OD_{600} reached 0.6, the culture was supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG; Nacalai Tesque, Japan) to a final concentration of 1 m*M* for induction. The cells were further cultured for 6 h at 310 K and were harvested by centrifugation. The cell pellet was suspended in resuspension buffer (50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl), sonicated and centrifuged. The pellet, including inclusion bodies, was washed with Triton buffer (0.5% Triton X-100, 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl) and with resuspension buffer. The purified inclusion bodies of PILRα were then dissolved in guanidine buffer (6 *M* guanidine–HCl, 50 m*M* MES–NaOH pH 6.5, 100 m*M* NaCl, 10 m*M* EDTA).

2.2. Protein refolding and purification

For refolding, 20 mg solubilized inclusion bodies of the human PILRα V-set domain were rapidly diluted into 1 l refolding buffer (1 *M* L-arginine–HCl, 0.1 *M* Tris–HCl pH 8.0, 2 m*M* EDTA) at 277 K. The solution was stirred at 277 K for 2 d and concentrated to 5–10 ml. The refolded protein was filtered (0.22 µm cutoff) and purified by gel-filtration chromatography (HiLoad 26/60 Superdex 75pg, GE Healthcare) with elution buffer comprising 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl. The PILRα protein eluted as a monomer, even though it has one free cysteine (Cys106; Fig. 1*a*). Further purification was performed by ion-exchange chromatography (Resource S, GE Healthcare) with elution buffer comprising 20 m*M* succinate pH 6.0, 0–200 m*M* NaCl. The purified PILRα protein was analyzed by SDS–PAGE with Coomassie Brilliant Blue (Qiagen) staining (Fig. 1*b*), which confirmed its high purity. Because PILRα is unstable at high pH, dialysis was conducted against 20 m*M* succinate pH 5.0, 100 m*M* NaCl buffer and the solution was concentrated to approximately 6 mg ml^{-1}. The selenomethionyl derivative of PILRα (referred to hereafter as SeMet PILRα) was prepared in the same manner, except that M9 medium containing selenomethionine (25 mg l^{-1}) was used instead of 2YT medium.

2.3. Crystallization

Initial crystallization trials were performed with Crystal Screens 1 and 2 (Hampton Research) and The Classics Suite (Qiagen) on Intelliplates (Art Robbins) using the automatic Hydra-Plus-One crystallization-setup robot (Art Robbins). The drop was a mixture of 0.2 µl protein solution (6 mg ml^{-1} PILRα, 20 m*M* succinate pH 5.0, 100 m*M* NaCl) and 0.2 µl reservoir solution and the crystallization plates were incubated with 90 µl reservoir buffer at 293 K. Crystals of native PILRα were obtained using The Classics Suite solution No. 7 (0.1 *M* trisodium citrate pH 5.6, 20% 2-propanol, 20% PEG 4000; Fig. 2*a*). However, the SeMet PILRα protein did not crystallize under the same conditions and extensive crystallization trials were therefore performed using the same commercially available screening kits as for the native PILRα. Crystals of the SeMet PILRα protein were successfully obtained using The PEGs Suite (Qiagen) solution No. 91 (0.2 *M* ammonium phosphate, 20% PEG 3350; Fig. 2*b*).

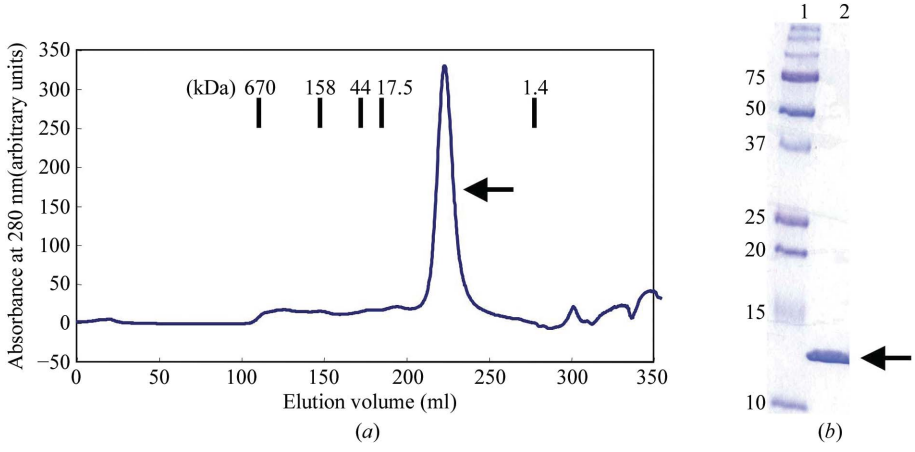
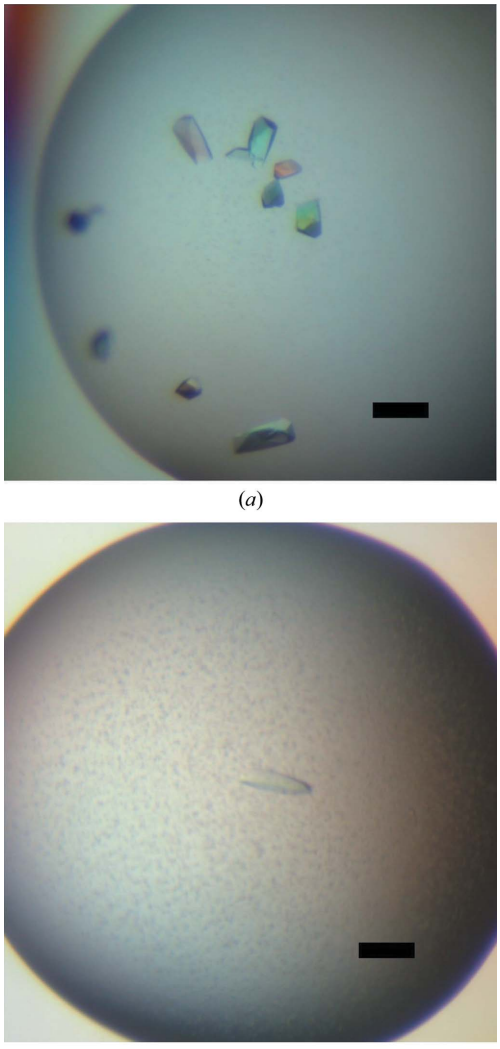


Figure 1
Gel-filtration chromatography and SDS–PAGE analysis of PILRα. (*a*) Gel-filtration chromatogram of PILRα. The arrow indicates the peak for the PILRα protein. (*b*) SDS–PAGE of the purified PILRα protein. Under reducing conditions, PILRα migrated as a 14 kDa band (the black arrow). Lane 1, molecular-weight markers (kDa); lane 2, PILRα protein after ion-exchange chromatography, ready for crystallization.



(*a*)

(*b*)

Figure 2
PILRα crystals grown by sitting-drop vapour diffusion. (*a*) Native crystals of PILRα. (*b*) SeMet PILRα crystals. The scale bars represent 0.1 mm.

2.4. X-ray diffraction analysis

Prior to data collection, the crystals of native PILRα were soaked in a cryoprotectant solution (0.1 *M* trisodium citrate pH 5.6, 20% 2-propanol, 30% PEG 4000) and flash-cooled. A 1.3 Å diffraction data set was collected at 100 K from the single largest crystal at beamline BL41 of SPring-8. Harima, Japan (λ = 0.9 Å). Data were processed and scaled with the *HKL*-2000 program package (Otwinowski & Minor, 1997). The PILRα structure could not be solved by molecular replacement using the crystal structure of sialoadhesin, which has 23% sequence similarity to PILRα, as a search model. Therefore, we prepared crystals of SeMet PILRα and MAD data sets were collected. However, this was also unsuccessful because of the low phasing power of the Se atoms (0.739 and 0.400 at the Se edge and peak, respectively). Finally, we prepared an iodide-anion derivative by soaking the native crystals in cryoprotectant solution including 1 *M* KI for 30 s prior to flash-cooling. A 1.8 Å diffraction SAD data set was collected at 100 K at beamline BL17A of the Photon Factory, Tsukuba, Japan (λ = 1.8 Å). The SAD data were processed and scaled with the *HKL*-2000 program package. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters

Table 1
Statistics of diffraction data collection and SAD phasing.

Values in parentheses are for the outer shell.

	Native	KI-soaked
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters		
a (Å)	40.33	40.77
b (Å)	44.94	45.51
c (Å)	56.87	55.94
X-ray source	BL41XU, SPring-8	BL17A, PF
Wavelength (Å)	0.9000	1.8000
Resolution (Å)	20–1.3 (1.33–1.30)	20–1.8 (1.86–1.80)
Observed reflections	183584 (17370)	67494 (6382)
Unique reflections	26166 (2555)	10063 (967)
Redundancy	7.0 (6.8)	6.7 (6.6)
Completeness (%)	99.9 (99.8)	99.4 (98.0)
$\langle I/\sigma(I)\rangle$	13.1 (4.1)	10.2 (7.56)
$R_{\rm merge}$ (%)†	5.8 (37.5)	8.6 (15.9)
ASU content	1 subunit	1 subunit
SAD phasing statistics		
Number of I sites in ASU		5
Phasing power (centric/acentric)		‡/2.486
⟨FOM⟩ (centric/acentric)		0.21311/0.57388

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\overline{I(hkl)}$ is the average intensity obtained from multiple observations of symmetry-related reflections. ‡ Not determined.

a = 40.4, b = 45.0, c = 56.9 Å, and contained one molecule per asymmetric unit. Five iodine sites were identified, refined and used for phase calculation with the *autoSHARP* package (Vonrhein *et al.*, 2006). The phases were further improved using the density-modification program *DM* (Cowtan, 1994) with a mean figure of merit of 0.574 for acentric reflections and of 0.213 for centric reflections. The statistics of data collection and SAD phasing are summarized in Table 1. The resulting electron-density map allowed the tracing of the main chain of the polypeptide. Model building and structure refinement are now in progress.

We thank S. Wakatsuki, N. Igarashi, N. Matsugaki, M. Kawamoto, H. Sakai, N. Shimizu and K. Hasegawa for assistance in data collection at the Photon Factory and SPring-8. This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Cowtan, K. (1994). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **31**, 34–38.
Diefenbach, A. & Raulet, D. H. (2003). *Curr. Opin. Immunol.* **15**, 37–44.
Fournier, N., Chalus, L., Durand, I., Garcia, E., Pin, J. J., Churakova, T., Patel, S., Zlot, C., Gorman, D., Zurawski, S., Abrams, J., Bates, E. E. & Garrone, P. (2000). *J. Immunol.* **165**, 1197–1209.
Lanier, L. L. (2001). *Nature Immunol.* **2**, 23–27.
Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., Biassoni, R. & Moretta, L. (2001). *Annu. Rev. Immunol.* **19**, 197–223.
Mousseau, D. D., Banville, D., L'Abbe, D., Bouchard, P. & Shen, S. H. (2000). *J. Biol. Chem.* **275**, 4467–4474.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Reid, S. W., Smith, K. J., Jakobsen, B. K., O'Callaghan, C. A., Reyburn, H., Harlos, K., Stuart, D. I., McMichael, A. J., Bell, J. I. & Jones, E. Y. (1996). *FEBS Lett.* **383**, 119–123.
Shiratori, I., Ogasawara, K., Saito, T., Lanier, L. L. & Arase, H. (2004). *J. Exp. Med.* **199**, 525–533.
Smith, H. R., Idris, A. H. & Yokoyama, W. M. (2001). *Immunol. Rev.* **181**, 115–125.
Vonrhein, C., Blanc, E., Roversi, P. & Bricogne, G. (2006). *Methods Mol. Biol.* **364**, 215–230.