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Crystallization and preliminary X-ray crystallographic analysis of macrophage/microglia-specific calcium-binding protein Iba1

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Iba1 (ionized calcium-binding adaptor molecule 1) is expressed specifically in macrophages/microglia and is involved in the activation of Rac, coordinating the reorganization of the actin cytoskeleton followed by the formation of lamellipodia and membrane ruffles, which are typical properties of activated microglia. Human Iba1 and mouse Iba1 were overexpressed and crystallized. The crystals belong to a monoclinic system, with space group $C2$ and unit-cell parameters a = 60.75, b = 36.61, c = 99.71 Å, β = 99.71° for human Iba1 and a = 76.28, b = 44.06, c = 99.13 Å, β = 90.03° for mouse Iba1. Both crystals diffracted well to a resolution of 2.1 Å and initial phase determinations were attempted by a molecular-replacement method using calmodulin structures.

1. Introduction

Microglia are glia cells in the brain and are one type of tissue-resident macrophage. In response to various environmental events, microglia are stimulated to exhibit morphological transformation, proliferation, migration and phagocytosis, and are transformed into so-called activated microglia (Streit & Kincaid-Colton, 1995; Kreutzberg, 1996; Nakajima & Kohsaka, 2001). Activated microglia are thought to act as neurosupportive cells, producing neuroprotective agents and scavenging cellular debris resulting from injury or disease. Therefore, the molecular mechanism of microglia activation is of great interest. Macrophage colony-stimulating factor (M-CSF) is considered to be one of the candidates responsible for maintaining the activation properties of microglia. In microglia stimulated by M-CSF, activated M-CSF receptor (Fms, tyrosine kinase receptor) causes the activation of phosphatidylinositol-3-kinase (PI3K); Rac (one of the Rho-family GTPases) is then activated by a classical PI3K-dependent signalling pathway. Activated Rac coordinates reorganization of the actin cytoskeleton, leading to the formation of lamellipodia and membrane ruffles, which are typical properties of activated microglia.

Iba1 (ionized calcium-binding adaptor molecule 1) has been identified as a novel calcium-binding protein which is expressed specifically in macrophages/microglia (Imai *et al.*, 1996; Ito *et al.*, 1998). Iba1 has been reported to be involved in the activation of Rac through a signalling pathway that differs from the well known PI3K-organized regulation of Rac (Imai & Kohasaka, 2002; Kanazawa *et al.*, 2002). The activated M-CSF receptor (Fms) activates phospholipase C-γ (PLC-γ) as well as PI3K; Iba1 and the activated PLC-γ then cooperate to activate Rac, coordinating the reorganization of the actin cytoskeleton followed by the formation of lamellipodia and membrane ruffles. Interestingly, Iba1 was also reported to have actin-binding and actin cross-linking activities, suggesting that Iba1 may directly interact with filamentous actin (Sasaki *et al.*, 2001). The three-dimensional structure of Iba1 will be very useful for understanding the molecular mechanism of microglia activation by the PLC-γ/Iba1-dependent signalling pathway. Here, we report the crystallization and preliminary X-ray analyses of human Iba1 (HIBA1) and mouse Iba1 (MIBA1).

2. Protein expression and purification

Using primers based on the sequences of a mouse Iba1 genome clone and the human cosmid 1.11 sequence (Iris *et al.*, 1993), mouse and human Iba1 cDNAs were amplified by PCR from J774A.1 and THP-1 cDNA pools, respectively (Ohsawa *et al.*, 2000). Iba1s fused with glutathione-*S*-transferase at an N-terminal site were expressed in *Escherichia coli* BL21 (DE3) using pGEX-4T vectors (Amersham). The procedures for purification of HIBA1 and MIBA1 were exactly the same. Cells were suspended in a buffer solution comprising 50 m*M* Tris–HCl pH 8.0, 5 m*M* EDTA, 1 m*M* PMSF, 5%(*w*/*v*) glycerol and 0.5 mg ml^{-1} lysozyme on ice. After 1 h, Triton X-100 was added to the suspension to a final concentration of 0.1%(*w*/*v*) and the suspension was left to stand for 30 min. Cells were disrupted by sonication for 1 min and cell debris was removed by centrifugation. The

supernatant was applied to a glutathione Sepharose 4B column (Amersham), which was washed with 0.1%(*w*/*v*) Triton X-100 (20 ml) in a buffer solution consisting of 10 m*M* phosphate pH 7.4, 100 m*M* NaCl and 2 m*M* KCl and subsequently with 20 ml of the same buffer solution without Triton X-100. The fusion protein bound to the matrix was cleaved with 10 units of thrombin (Sigma) for 18 h at 281 K. The eluted fraction was loaded onto a gel-filtration column (Superdex 200, Amersham). The purity of the final sample was checked by SDS–PAGE.

3. Crystallization

Crystals of HIBA1 and MIBA1 were obtained by the hanging-drop vapour-diffusion method. An initial screening of crystallization conditions using Crystal Screens 1 and 2 (Hampton Research) was attempted using 96-well plates (Corning). The final crystallization procedures for HIBA1 and MIBA1 were as follows. HIBA1 (10 mg ml^{-1}) in 5 m*M* Tris–HCl buffer pH 7.0 was mixed with an equal amount of the reservoir solution containing 20%(*w*/*v*) polyethylene glycol (PEG) 4000, 10%(*w*/*v*) 2-propanol and 0.01 *M* $NiCl_2$ in 100 m*M* Tris–HCl pH 7.3. 10 µl crystallization drops were equilibrated against 1 ml of the reservoir solution at 293 K using 24-well plates (Corning). A prismatic crystal of dimensions 0.1 × 0.1 × 0.05 mm grew after two weeks (Fig. 1*a*). A prismatic crystal of MIBA1 with dimensions 0.1 × 0.1 × 0.05 was obtained from a reservoir solution consisting of 1.4 *M* sodium citrate in 100 m*M* Na HEPES pH 7.5 at 293 K by the same procedure (Fig. 1*b*).

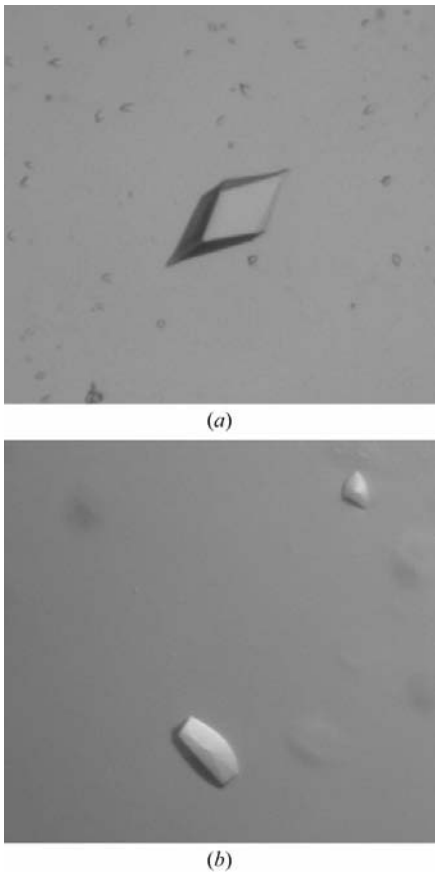

Figure 1
Crystals of (*a*) HIBA1 and (*b*) MIBA1.

4. Data collection and processing

Data collection for HIBA1 and MIBA1 were carried out using synchrotron radiation on beamline NW-12 at the Photon Factory (Tsukuba, Japan) under the approval of the Photon Factory Advisory Committee and the National Laboratory for High Energy Physics, Japan (proposal 2003S2-002). Crystals were removed using crystal loops (Hampton Research) and immediately frozen in a stream of evaporating nitrogen. Diffraction data were then collected using an ADSC/CCD detector system at 100 K.

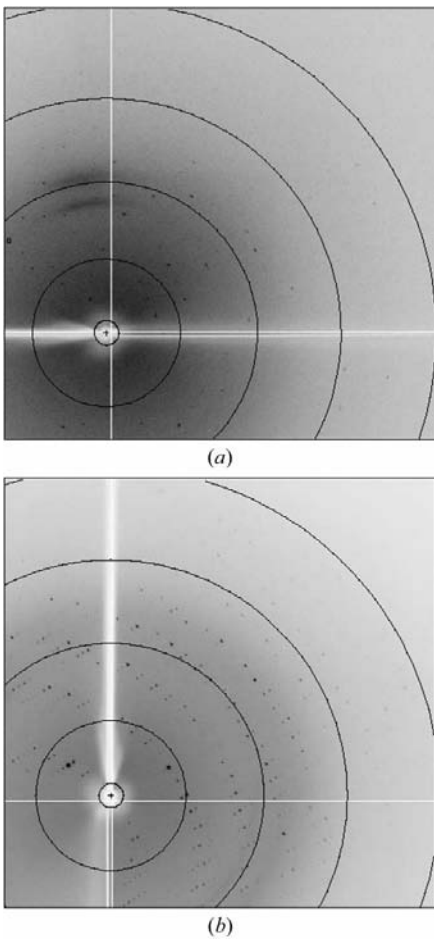

Figure 2
Diffraction images of (*a*) HIBA1 and (*b*) MIBA1 with a resolution scale (7.6, 3.8, 2.5 and 1.9 Å).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.10–2.18 Å).

	HIBA1	MIBA1
Beamline (Photon Factory)	NW12	NW12
Wavelength (Å)	1.0	1.0
Temperature (K)	100	100
No. of crystals	1	1
Resolution (Å)	2.10	2.10
No. measured reflections	40318	46153
No. unique reflections	5679	16909
$R_{\rm merge}$†	0.073 (0.151)	0.060 (0.197)
Completeness (%)	100 (100)	86.7 (58.3)
$I/\sigma(I)$	14.4	11.5
Space group	*C*2	*C*2
Unit-cell parameters		
a (Å)	60.75	76.28
b (Å)	36.61	44.06
c (Å)	44.02	99.13
β (°)	99.71	90.03
Volume (Å^3)	96500	333166
MW (Da)	16703	16910
No. of molecules in AU	1	2
$V_{\rm M}$ (Å^3 Da^{-1})	1.44	2.46

† $R_{\rm merge} = \sum\sum|I_i - \langle I\rangle|/\sum\langle I\rangle$.

Data processing was carried out using the program *HKL*2000 (Otwinowski & Minor, 1997) on the beamline station. The data-collection statistics for HIBA1 and MIBA1 are listed in Table 1.

5. Results and discussion

Crystals of HIBA1 and MIBA1 diffracted well to a resolution of 2.1 Å, as shown in Fig. 2, which is sufficient to reliably determine the crystal structure. HIBA1 and MIBA1 contain 147 amino-acid residues with 90% sequence homology, including two EF-hand motifs (Asp58–Met73 and Ser94–Met109), which are common among calcium-binding proteins. The region comprising the two EF-hand motifs of MIBA1 (Phe57–Lys114) has 33% sequence homology with mouse calmodulin, while the N- and C-terminal regions sites both show no homology with any known protein (Imai & Kohasaka, 2002). We attempted to determine the initial phases of HIBA1 and MIBA1 by a molecular-replacement method using the calmodulin structures in the PDB (Berman *et al.*, 2000) as probe models. However, a cross-rotation function with PC refinement calculated using the program *CNS* (Brünger *et al.*, 1998) did not give a clear solution in any of the trials. A search for suitable heavy-atom derivatives and the preparation of crystals of selenomethionine-substituted proteins are in progress.

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