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Expression, purification and characterization of leukotriene B_4 receptor, BLT1 in *Pichia pastoris*

Tetsuya Hori [a], Yo Sato [a], Naoko Takahashi [a], Koji Takio [a], Takehiko Yokomizo [b], Motonao Nakamura [c], Takao Shimizu [c], Masashi Miyano [a,*]

[a] Structural Biophysics Laboratory, RIKEN SPring-8 Center, Harima Institute, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan
[b] Department of Medical Biochemistry, Graduate School of Medical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan
[c] Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

The high yield expression of BLT1, a G-protein coupled receptor for leukotriene B_4, was established in *Pichia pastoris* for structural studies. Guinea pig BLT1 was expressed in a functional form without post-translational modifications for the rapid purification and the crystallization. Among the BLT1s from four species, only guinea pig BLT1 was successfully expressed with the comparable binding affinity to BLT1 of native guinea pig tissues for several ligands. Only Asn4 of the two putative *N*-glycosylation sites was glycosylated, and the mutation to Ala to avoid glycosylation did not affect the ligand binding affinity. However, the N-terminal region of the mutant was digested at the carboxyl ends of Arg3 and Arg8, as detected by N-terminal amino acid sequencing, and Ser309 in the C-terminal region was partially phosphorylated, as identified in the micro-sequencing by Q-TOF-MS/MS. To avoid chemical heterogeneity, the N-terminal peptide (1–14) truncated and the C-terminal phosphorylation-site eliminated mutant was generated. The binding affinity of the mutant's membrane fraction for LTB_4 was K_d = 6.6 nM and B_{max} = 50.0 pmol/mg membrane protein. The yield of purified mutant was approximately 0.3–0.4 mg from 1 L culture, and the protein showed a single peak at molecular weight of 100 kDa in gel-filtration and no glycosylation or phosphorylation in MALDI-TOF MS.

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Introduction

Leukotriene B_4 (LTB_4; 5[*S*],12[*R*]-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid)[1] is a lipid mediator enzymatically metabolized from arachidonic acid of membrane phospholipids in cells at inflammation regions [1]. LTB_4 induces a chemotaxis of leukocytes such as granulocytes and macrophages in the initial responses of inflammation [2]. LTB_4 also induces a migration of both $CD4^+$ and $CD8^+$ T cells to tissues in the early phase of the immune response [3–5]. The extracellular LTB_4 signal is transmitted to the LTB_4 receptor (BLT1) of the target cells [6]. BLT1 is a rhodopsin-family G-protein coupled receptor (GPCR) and activates the intracellular cascade reactions via G(i)-family and G(16) G-proteins [7]. It is thought that accumulation of LTB_4 is involved in various inflammatory diseases, such as asthma, rheumatoid arthritis and chronic obstructive pulmonary disease [8,9]. BLT1 antagonists have been developed for therapeutics of these inflammatory diseases [10]. The crystal structure of BLT1 is indispensable for drug discoveries.

For the crystallographic studies, the preparation of a large quantity of homogeneously purified BLT1 with a functionally folded conformation and the least chemical heterogeneity is crucial for growing suitable crystals. Over-expressions of BLT1 were reported in *Escherichia coli* [11] and *Spodoptera frugiperda* (*Sf*9) cell – baculovirus particle system [12]. In *E. coli* using the codon optimization gene, the expression level was reported to be mgs per 1 L culture as inclusion body, which was refolded as active form by Banères et al. [11], and their substantial improvements of the refolding functional BLT1 using detergent with novel supplementary material Amphipol have been continued including its characterization for structural studies in recent studies by Banères et al. [13]. A large scale preparation of BLT1 in *E. coli* does not have post-translational modification including glycosylation and/or phosphorylation due to the nature of *E. coli* system, but it remained to be a monomodal sample in gel chromatography by refolding from inclusion body of BLT1 peptide [13]. Whereas, the over-expression as inclusion body peptide has not been successful using the simpler approaches in the *E. coli* Rosetta™ strain [14] and the synthesizing full-length cDNA substituted all rare codons for *E. coli* according to the

* Corresponding author. Fax: +81 791 58 2816.
E-mail address: miyano@spring8.or.jp (M. Miyano).

[1] *Abbreviations used:* BSA, bovine serum albumin; GPCR, G-protein coupled receptor; LTB_4, leukotriene B_4 (5[*S*],12[*R*]-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid); DDM, dodecyl-β-D-maltoside; αPP, prepro sequence of a *Saccharomyces cerevisiae* α-factor; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue.

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description by Banères et al. [11] (Hori et al., unpublished data). Additionally, the expression of human BLT1 by isolated budding baculavirus particles from *Sf*9 culture medium was high (B_{max} = 27 pmol/mg membrane protein) [12], but the baculavirus particles expression system is not suitable for large scale preparation like as common *Sf*9/baculavirus system. Therefore, we have adopted to establish the high expression of the functional BLT1 with less post-translational modifications using the methylotrophic yeast *Pichia pastoris* [15] other than *E. coli* and the *Sf*9 – baculovirus particles as alternative choice, since the structural analysis of GPCR expressed in yeast is challenge because of no structural reports of GPCRs despite of intensive works [15].

In this study, the glycosylation- and phosphorylation-deficient form of guinea pig BLT1 was successfully overexpressed in *P. pastoris* as the N-terminal truncated and phosphorylation-site eliminated mutant with high expression level (B_{max} = 50 pmol/mg membrane protein), and the purified protein was obtained 0.38 mg from 1 L culture. The purified mutant showed a single and symmetrical elution profile in a gel-filtration with apparent molecular weight of 100 kDa and was confirmed to be without glycosylation or phosphorylation in MALDI-TOF/MS analyses and N-terminal peptide sequencing. Due to the lack post-translational modifications, it allows a rapid and simple purification of BLT1, and it is advantageous for further structural studies.

Materials and methods

Materials

Expression vectors, transformation reagents and the GS115 strain of *P. pastoris*, the Yeast Nitrogen Base and anti-mouse antibody conjugated with horseradish peroxidase were purchased from Invitrogen. Endoglycosidase H (Endo-H), and Peptide:*N*-glycosidase F (PNGase-F) were from Roche Diagnostics. Restriction enzymes and agarose were purchased from TaKaRa Bio. *E. coli* strain DH5α, Ligation Hi, alkaline phosphatase and KOD DNA polymerase were purchased from TOYOBO. The QuikChange kit was purchased from Stratagene. Oligo DNAs were from Hokkaido System Science. Yeast extract was purchased from Merck. Peptone, tryptone and TALON resin were purchased from BD biosciences. Agar was purchased from Kishida Chemical. The plasmid preparation kit and the DNA fragment extraction kit from agarose gel were purchased from QIAGEN. Dodecyl-β-D-maltoside (DDM) was from Anatrace. Purification reagents, skim milk, bovine serum albumin (BSA) for BCA assay, histidine, methanol, glycerol, acetonitrile and trifluoroacetic acid were from Wako Pure Chemical. BCA assay reagents were purchased from Pierce. ECL solution, Superose-12 column, PD-10 column, Q-Sepharose, G25 desalt resins and XK empty column were purchased from GE healthcare. Molecular weight marker for SDS–PAGE, empty columns, anti-mouse antibody conjugated with alkaline phosphatase, colorimetric alkaline phosphatase substrate reagents and an electrophoretic transfer cell for Western blotting were purchased form Bio-Rad. SDS–PAGE equipment was purchased from ATTO. BSA for ligand-binding assay, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), anti-His antibody, anti-FLAG antibody, anti-FLAG agarose gel, FLAG peptide and biotin were purchased from Sigma. The ultra-filtration device was purchased from Millipore. DNA and peptide sequence reagents were purchased from Applied Biosystems. The molecular weight marker for MALDI-TOF was from Bruker Daltonics. Glass beads were from BioSpec. ^{3}H-LTB$_4$ was purchased from Perkin-Elmer Life Sciences. LTB$_4$ was purchased from Cayman Chemicals. BIIL 260 and BIIL 284 were generously donated by Boehringer Ingelheim. CP105,696 was a gift from Pfizer Inc. ZK158252 was a gift from Bayer Schering Pharma AG.

Construction of the expression vectors

All the primers, oligo DNAs, PCR templates and restriction enzymes used for vector constructions are summarized in Supplemental Table S1. The cDNAs of BLT1 (GenBank Accession Nos.: AB005049.1, guinea pig; BC064063.1, mouse; AB025230.1, rat; AB008193.1, human) were amplified by PCR and ligated into each expression vector. The mutations of N4A, N165A, N165Q and S309A were introduced using a QuikChange kit (Stratagene). All the DNA sequences of the ORF region of the expression vectors were verified with a DNA sequencer ABI Prism 310 (Applied Biosystems).

Culture medium for yeast manipulation and expression (Invitrogen)

BMGY: 1% yeast extract, 2% peptone, 0.1 M potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 1% glycerol, 4×10^{-5}% biotin. BMMY: the same as BMGY with 0.5% methanol instead of glycerol. BMMH: the same as BMMY without yeast extract and peptone. YPD: 1% yeast extract, 2% peptone, 2% dextrose. MD agar plate: 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% dextrose, 1.5% agar.

Buffers for expression and purification

Breaking buffer: 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5% glycerol. MS buffer: 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% DDM. Stripping solution: 100 mM Tris–HCl (pH 6.8), 2% SDS and 100 mM 2-mercaptoethanol. Glycosidase buffer: 100 mM K-Phosphate buffer (pH 6.0) and 1% DDM.

Transformation of expression vectors into P. pastoris and small scale expression of BLT1

The expression vectors linearized with Sac I were transformed into the *P. pastoris* GS115 strain on MD agar plates using the *Pichia* EasyComp kit (Invitrogen). The BLT1 expression was examined with the small scale expression from a single colony of the His$^+$ transformant (Invitrogen). The expressed cells were suspended with 1 ml of the breaking buffer. The cells were broken with 300 μl of glass beads (0.5 mm) in 2 ml tube by twelve 30 s vortex applications and a cooling cycle on ice-cold water. The cell-broken slurry was centrifuged at 800*g* for 5 min at 4 °C. The collected supernatants were ultra-centrifuged at 100,000*g* for 60 min at 4 °C. The pellets were re-suspended in 100 μl of the ice-cold breaking buffer and sonicated for 0.5 s five times by a sonicator as the membrane fraction. For the preparation of the glycerol stock of clones, the collected cells after overnight culture were suspended in YPD medium containing 15% glycerol and the suspension was frozen with liquid nitrogen and stored at −80 °C as stock clones.

Large scale expression of BLT1

Colonies were re-generated from the glycerol stock on a MD plate at 30 °C for at least 2 days. The cells were cultured from the colonies with 10 ml, 400 ml and 1 L BMGY medium at 30 °C as subcultures. Typically, the culture reached $OD_{600\ nm}$ = 14. The medium was replaced by 1 L of induction medium after centrifugation at 3500*g* for 15 min at 20 °C. In the case of the lower cell density culture, which was started with the induction at the early log-phase, the initial cell density was adjusted to $OD_{600\ nm}$ = 1.0. The BLT1 expression was induced with shaking at 100 rpm for 24 h in a 5 L baffled flask. The induction medium (BMMY or BMMH) and temperature (30 or 20 °C) were optimized for each mutant. The cells were harvested by centrifugation at 3500*g* for 15 min at 4 °C, washed with de-ionized water and stored at −30 °C until use.

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