Protein Expression and Purification 77 (2011) 86–91

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Expression and purification of human FROUNT, a common cytosolic regulator of CCR2 and CCR5

Kaori Esaki [a], Yuya Terashima [b,c], Etsuko Toda [c], Sosuke Yoshinaga [a], Norie Araki [a], Kouji Matsushima [c], Hiroaki Terasawa [a,*]

[a] Faculty of Life Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
[b] Central Lab, ECI Inc., Meguro-ku, Tokyo, Japan
[c] Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

ARTICLE INFO

Article history:
Received 16 November 2010
and in revised form 19 December 2010
Available online 28 December 2010

Keywords:
GPCR
Chemokine receptor
FROUNT
pCold
Trigger factor

ABSTRACT

Chemokine receptors play pivotal roles for immune cell recruitment to inflammation sites, in response to chemokine gradients (chemotaxis). The mechanisms of chemokine signaling, especially the initiation of the intracellular signaling cascade, are not well understood. We previously identified a cytoplasmic protein FROUNT, which binds to the C-terminal regions of CCR2 and CCR5 to mediate chemokine signaling. Although large amounts of purified protein are required for detailed biochemical studies and drug screening, no method to produce recombinant FROUNT has been reported. In this study, we developed a method for the production of recombinant human FROUNT. Human FROUNT was successfully expressed in *Escherichia coli*, as a soluble protein fused to the folding chaperone Trigger Factor, with a cold shock expression system. The purified FROUNT protein displayed CCR2 binding ability without any additional components, as demonstrated by SPR measurements. A gel filtration analysis suggested that FROUNT exists in a homo-oligomeric state. This high-yield method is cost-effective for human FROUNT production. It should be a powerful tool for further biochemical and structural studies to elucidate GPCR regulation and chemokine signaling, and also will contribute to drug development.

Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of membrane proteins and mediate a multitude of cellular and physiologic responses to specific ligands [1]. Mutations in the genes encoding GPCRs are implicated in numerous diseases, and these receptors presently form the largest class of therapeutic targets [2]. Chemokine receptors play pivotal roles for immune cell recruitment to inflammation sites, in response to chemokine gradients (chemotaxis). This innate immune system is absolutely required for host defense, although when it becomes uncontrolled, it leads to inflammatory disease. Approximately 20 plasma membrane receptors have been characterized as members of the chemokine receptor family, and all of them are GPCRs [3].

Mutational analyses revealed that the cytoplasmic C-terminal domain, especially the membrane-proximal C-terminal region (Pro-C), of chemokine receptors plays an important role in chemotaxis [4–9]. In the cases of CCR2 and CCR5, the truncation of the Pro-C also impairs the chemokine signals, without the loss of cell surface localization [4,5]. We previously identified a 75-kDa cytoplasmic protein, FROUNT, which interacts with the Pro-C regions of CCR2 and CCR5, using a yeast two-hybrid system [10,11]. FROUNT directly binds to activated CCR2 and CCR5 and mediates directional cell migration. Since FROUNT does not bind to the C-terminal regions of CCR1, CCR3 and CXCR4, it was suggested that FROUNT interacts specifically with CCR2 and CCR5 [11]. The mechanisms of chemokine signaling, and especially the initiation of the intracellular signaling cascade, are not well understood. Since FROUNT lacks homology with known GPCR regulators, FROUNT may mediate the chemokine signaling in a novel manner. Clarification of the function of FROUNT will provide new insights into chemokine signaling and general GPCR regulation.

CCR2 and CCR5 are involved in various diseases, including chronic inflammation, cancer progression and viral infection, and thus FROUNT is considered as a promising drug target to treat a wide range of diseases. Various reports have indicated that FROUNT could actually have effective therapeutic applications: (1) We previously reported that macrophage infiltration was inhibited by FROUNT depletion, in a mouse peritonitis model [10]. (2) Belema-Bedada et al. reported that FROUNT is required for the migration and recruitment of CCR2-expressing bone marrow-derived mesenchymal stem cells to injured heart tissue [12]. (3) Satoh et al. showed that the mRNA levels of both FROUNT and

* Corresponding author. Fax: +81 96 371 4310.
E-mail address: terasawa@gpo.kumamoto-u.ac.jp (H. Terasawa).

1046-5928/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2010.12.012

CCR2 were up-regulated in biopsy tissue samples from patients with heart failure [13]. (4) Golen et al. reported that FROUNT mediates the transendothelial migration of prostate carcinoma cells [14].

Although large amounts of purified protein are required for detailed biochemical studies and drug screening, no method to produce recombinant FROUNT has yet been reported. We report here the first successful expression and purification of human FROUNT. We expressed human FROUNT fused to Trigger factor (TF), by a cold shock expression system in *Escherichia coli* (*E. coli*). The purified FROUNT protein retained the bind ability to CCR2. A gel filtration analysis suggested that FROUNT has oligomeric properties.

Materials and methods

Materials

Restriction enzymes were purchased from Toyobo Co., Ltd. PrimeStar DNA[1] polymerase and pCold TF DNA were purchased from Takara Bio Inc. SYBR® Safe DNA gel stain was purchased from Invitrogen. Molecular weight standards for SDS PAGE were purchased from BioRad. The Gel Filtration Calibration Kit (HMW) was purchased from GE Healthcare. The synthesized peptides, CCR2 Pro-C (EKFRRYLSVFFRKHITKRF) and 3 × FLAG peptide (DYKDDDDKDYKDDDDKDYKDDDDK), were purchased from Hokkaido System Science Co., Ltd. Other reagents were purchased from Nacalai Tesque, Inc. and Wako Chemicals, unless otherwise noted.

Construction of expression vectors

The DNA fragment encoding human FROUNT was amplified by polymerase chain reaction (PCR) and cloned in-frame into the pCold TF DNA vector, between the *Bam*HI and *Sal*I sites. A TEV protease site (ENLYFQG) was inserted just before the human FROUNT gene, by site-directed mutagenesis. The resulting plasmid, named pTF-FNT, generates the FROUNT protein fused with a hexahistidine-tag (His_6-tag) and Trigger factor (TF) at the N-terminus.

Protein expression

The His_6-TF-FROUNT fusion protein was expressed in *E. coli* BL21-CodonPlus™-RP cells (Stratagene) transformed with pTF-FNT. The cells were grown to an OD_{600} of 0.5 at 37 °C, in M9-tryptone medium (12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 10 g tryptone (BD) per liter of H_2O, 1 mM $MgSO_4$, 0.1 M $CaCl_2$, 0.5% glucose, 32 μg/mL chloramphenicol, 100 μg/mL ampicillin) [15]. To induce His_6-TF-FROUNT fusion protein production, 0.1 mM isopropyl-thio-galactopyranoside (IPTG) was added to the culture, which was incubated for an additional 12 h at 15 °C. The cells were collected by centrifugation at 4800 × *g* at 4 °C for 15 min and frozen at −80 °C.

Protein purification

The cell pellet (4 g) was resuspended in 40 mL of A buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM DTT), containing 1 mL of Protease Inhibitor Cocktail (Nacalai Tesque, Inc.), and lysed by sonication. Brij-35 was added to the cell lysate at a final concentration of 0.03%, and then it was centrifuged at 30,000 × *g* at 4 °C for 15 min. The supernatant was applied to a 10 mL Ni Sepharose 6 Fast Flow (GE Healthcare) column, pre-equilibrated in A buffer containing 0.03% Brij-35. The column was washed with 50 mL of B buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.03% Brij-35, 200 mM imidazole), and the fusion protein was eluted with 20 mL of C buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.03% Brij-35, 200 mM imidazole). TEV protease (AcTEV™ Protease, Invitrogen) was added to the His_6-TF-FROUNT fusion protein, and the solution was dialyzed against D buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, 0.03% Brij-35) at 4 °C for 24 h. The digested sample was loaded on a nickel-affinity column packed with 10 mL of Ni Sepharose 6 Fast Flow resin, to remove the histidine-tagged contaminants, including the uncleaved fusion protein, His_6-TF and TEV protease. The flow-through fraction was further purified by gel-filtration chromatography (HiLoad 26/60, Superdex 200 prep grade, GE Healthcare).

MALDI-TOF MS analysis of the intact protein

The solution containing the purified FROUNT protein was desalted, using a ZipTip C18 pipette tip (Millipore). The MALDI-TOF analysis was performed on a Bruker ultrafleXtreme (Bruker Daltonics) mass spectrometer. The sample (1 μL) was mixed with an equal volume of sinapic acid matrix solution in 50% acetonitrile and 0.1% TFA, and was spotted onto the target plate. Bovine serum albumin was used for calibration. For the average masses obtained in the linear mode, the mass accuracy was set at ~10 ppm.

Limited proteolysis

Recombinant FROUNT was digested with trypsin and chymotrypsin in A buffer at 37 °C for 20 min. The weight ratios of proteases to FROUNT were 1:100 and 1:10. The reactions were quenched by the addition of 0.1% TFA, and the proteolytic products were lyophilized.

N-terminal amino acid sequencing

Intact and digested FROUNT preparations were fractionated on a 15% SDS–PAGE gel and electrotransferred to a PVDF membrane. The bands were excised from the membrane after staining with Coomassie Brilliant Blue R250. After washing with methanol and Milli-Q water, the membrane pieces were analyzed by a protein sequencer (Procise® HT, Applied Biosystems) to determine the N-terminal amino acid sequences of intact and digested FROUNT.

In-gel digestion

Lyophilized samples were fractionated on a 15% SDS–PAGE gel, and the bands were excised from the gel after staining with a Rapid Stain CBB Kit. The gel pieces were diced into about 1 mm^3 pieces, and were digested according to the procedure reported by Ochi et al. [16], with minor modifications. The gel pieces were treated with 50 mM ammonium bicarbonate in 100% (v/v) acetonitrile and vacuum-dried. Sequence Grade Modified Trypsin (Promega) was added at a concentration of 50 μg/mL to the gel pieces, in 6.6% acetonitrile including 50 mM ammonium bicarbonate, and the mixture was incubated at 37 °C O/N. The trypsinized peptides were sequentially extracted from the gels with 0.1% (v/v) TFA in 30% (v/v) acetonitrile, 50% (v/v) acetonitrile, and 80% (v/v) acetonitrile, for 5 min each. The extracted peptides were vacuum-dried, dissolved in 20 μL of 0.1% (v/v) TFA, and desalted with a ZipTip C18 pipette tip.

[1] Abbreviations used: DNA, deoxyribonucleic acid; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; OD, optical density; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; Tris, tris (hydroxymethyl) aminomethane.