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Over-expression and purification of isotopically labeled recombinant ligand-binding domain of orphan nuclear receptor human B1-binding factor/human liver receptor homologue 1 for NMR studies

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Abstract

The human hepatitis B virus enhancer II B1 binding factor (hB1F), which regulates the expression of hepatitis B virus genes, is identified as a nuclear receptor. It regulates several liver-specific genes and plays an important role in the bile acid biosynthesis pathway. A significantly optimized protocol has been worked out to prepare ^{15}N and/or ^{13}C-labeled hB1F ligand-binding domain in minimal medium with high yields for NMR studies. Under the various conditions optimized for the purification of His_6-hB1F ligand-binding domain, the yield of the purified protein is estimated to be 25–30 mg from 0.5 L of M9 minimal media. Electrospray ionization mass spectrometry data confirm the correctness of the primary sequence. Dynamic light scattering experiment proves that the protein exists as a monomeric form. In addition, the circular dichroism results show that the protein has a well-regulated secondary structure and a high α-helical content in ammonium bicarbonate buffer at 20 °C and pH 7.4. Finally, uniformly ^{15}N-labeled protein is characterized by a TROSY–HSQC spectrum, and the dispersion of ^{15}N–^{1}H cross-peaks in the spectrum indicates the presence of well-ordered and properly folded protein as a monomer.

Keywords: hB1F/hLRH-1; Isotope labeling; M9 minimal medium; NMR; NR5A2; Protein over-expression; Purification; Recombinant LBD

Nuclear receptors (NRs)[1] are one of the largest families of transcription factors having 48 members identified in the human genome. The NR superfamily has been divided into seven subfamilies (NR0–NR6) [1]. Members of this superfamily include receptors for steroid and non-steroid hormones, as well as a large number of orphan receptors whose regulatory ligands have not been identified [2,3]. Generally, the members of the nuclear receptor superfamily bind to DNA response elements as a hetero- or homodimer and regulate gene

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[1] *Abbreviations used:* DLS, dynamic light scattering; ESI-MS, electrospray ionization mass spectrometry; FT, Fourier transform; hB1F, human hepatitis B virus enhancer II B1 binding factor; HBV, hepatitis B virus; hLRH-1, human liver receptor homologue 1, HSQC, heteronuclear single quantum correlation; IEF, isoelectric focusing gel electrophoresis; IPTG, isopropyl β-D-thiogalactoside; LBD, ligand-binding domain; NMR, nuclear magnetic resonance; NR, nuclear receptor; PMSF, phenylmethylsulfonyl fluoride; TROSY, transverse relaxation-optimized spectroscopy.

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transcription in association with a variety of cofactors. *Fushi tarazu* factor (Ftz-F1) is one of the seven subfamilies of the NR superfamily [4], and is nominated as NR5A [5]. The members of the Ftz-F1 subfamily all possess a particular Ftz-F1 box located at the C-terminal of the DNA-binding domain (DBD) and bind to their response elements as a monomer [6]. They have been identified in metazoan ranging from *Caenorhabditis elegans* to human and proved imperative for proliferation and development [7–9].

Human B1-binding factor (hB1F, also known as NR5A2, hLRH-1, FTF or CPF) is a novel hepatocyte transcription factor, which binds specifically to the B1 element in Enhancer II (EN II) of hepatitis B virus (HBV) and activates the functions of EN II, thus regulate the expression of HBV genes. Based on the sequence similarity, hB1F is identified as an orphan nuclear receptor belonging to the FTZ-F1 subfamily [10]. Previous studies showed that hB1F is expressed specifically in liver, pancreas, intestine, ovary, adrenal, and preadipocytes [11–14]. hB1F plays an important role in bile acid and cholesterol homeostasis by regulating several key enzymes and transporters [15–19]. It can also modulate the expression of aromatase [14] and several liver-enriched transcriptional factors such as HNF1α, HNF3β, and HNF4α [20]. In addition, as we have discussed before, hB1F can activate HBV enhancer II, which in turn regulates hepatic viral gene expression and replication [10,21].

Like other nuclear receptors, hB1F is composed of several modular functional domains including an N-terminal A/B domain, DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD). As we know, most of nuclear receptors are ligand-dependent transcription factors mediating the action of lipophilic hormones and are the targets of drugs useful in a variety of therapeutic area [22]. Thus LBD is a functionally important part, to which close attention has been paid in recent years. Recently, the X-ray crystal structures of mLRH-1 LBD and hLRH-1 LBD have been reported [23,24]. Although many studies about hB1F have been reported recently, the functional studies of hB1F are still in progress [25–29]. Recently, Redinbo and co-workers [30] reported the crystal structure of the LBD of hLRH-1 in complex with the NR box 1 motif of human SHP and revealed that hLRH-1 controls gene expression in response to phospholipids, and thus may be effectively targeted by designed small molecules. For the study on the interaction of the protein with various natural or synthesized small molecules, the NMR structure of the molecule in solution with the valuable dynamic information is desirable. However, so far no NMR structure of hB1f/hLRH-1 LBD in solution has been reported. In this endeavor, we have initiated to unravel the three-dimensional (3D) structure at atomic resolution of the isotopically labeled hB1F_LBD in solution by multidimensional NMR spectroscopy. In this paper, we report a significantly optimized protocol to prepare isotopically (^{15}N and/or ^{13}C) labeled hB1F_LBDin minimal medium with high yields. The conditions necessary to stabilize the structure of this protein for NMR experiments are also discussed.

Materials and methods

Construction of plasmid pEThb1flbd

The plasmid pQE32CL is a pQE32 vector (Qiagen) containing the cDNA segment 450–1488 of *hb1f*, which includes a fragment coding for the LBD (residues 290–541) of hB1F [31]. The *hb1flbd* gene fragment was amplified from the pQE32CL by PCR using a forward primer (5′-GGAATTCAT**ATG**GATAGTTACCAGACGAG CTCTC-3′) including an *Nde*I site (underline), which contains the start codon ATG (bold), and a reverse primer (5′-CCCAAGCTTCTGCAG**TTA**TGCTCTTT TGGCATGCAA-3′), which contains a *Hin*dIII site (underline), followed by the minus part of a stop codon TAA (bold). The PCR protocol for 30 cycles was as follows: 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min. *Taq* DNA polymerase (Biolab) was used. This amplified fragment after digestion by *Nde*I/*Hin*dIII was inserted into *Nde*I/*Hin*dIII-digested vector, pET-28a (+) (Novagen) to construct an expression plasmid pEThb1flbd. All DNA recombinant manipulations were performed according to the standard protocol [32]. The DNA sequencing was achieved on a DNA sequencer by Shanghai Genecore Biotechnologies.

Protein expression and preliminary identification

Transformation of a host cell *Escherichia coli* BL21 (DE3) (Novagen) by plasmid pEThb1flbd was carried out with the routine procedure. A few transformants from the LB media agar plates containing 30 μg/ml of kanamycin were picked out to inoculate in 5 ml of LB medium with the same antibiotics and cultured at 37 °C overnight. Then the culture was diluted 100-fold with same LB medium and cultured at 37 °C to A_{600} of 0.6–0.7, followed by induction expression with IPTG (final concentration 1 mM) for 0.5, 1, 4, and 8 h. After being lysed by the routine procedure, the samples were loaded onto a 15% gel for SDS–PAGE to screen for the right transformants showing high level expression of hB1F-LBD.

Preparative over-expression of hB1F-LBD in M9 minimal medium

An overnight culture containing the right transformant BL21 (DE3, pEThb1flbd) was treated as above in 5 ml LB