Biochemical and Biophysical Research Communications 426 (2012) 260–265

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

A new method for establishing stable cell lines and its use for large-scale production of human guanylyl cyclase-B receptor and of the extracellular domain

Naoki Okamoto, Haruo Ogawa, Chikashi Toyoshima *

Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

ARTICLE INFO

Article history:
Received 12 August 2012
Available online 23 August 2012

Keywords:
Guanylyl cyclase-B receptor
C-type natriuretic peptide
Stable expression
Purification

ABSTRACT

Guanylyl cyclase-B receptor (GC-B) is a membrane receptor that induces intracellular accumulation of cGMP when a specific ligand, C-type natriuretic peptide (CNP), binds to the extracellular ligand-binding domain (ECD). Despite of its medical and biological importance, characterization of GC-B is hampered by limited amounts of protein obtainable. To circumvent this problem, a method was developed for rapidly and semi-automatically establishing stable cell lines specialized for large-scale production. This method, utilizing a bicistronic expression vector for co-expressing a green fluorescent protein and FACS-based selection of high-expressing cells, is generally applicable. It worked particularly well with the ECD and yielded highly purified ECD at 1 mg/l of culture medium by affinity chromatography using modified CNPs. Measurements of ligand-binding and guanylyl cyclase activities for various natriuretic peptides showed that, as expected, CNP is by far the most potent agonist of GC-B with IC_{50} of ~7.5 nM. This value is at least an order of magnitude larger than that reported earlier but similar to that established with the guanylyl cyclase-A receptor for its ligand, atrial natriuretic peptide. The methods developed here will be useful, at the least, for characterizing other members of the guanylyl cyclase receptor family.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Guanylyl cyclase-B receptor (GC-B) is a member of the guanylyl cyclase (GCase)-coupled receptor family that comprises seven subtypes (termed GC-A through GC-G) in mammalian tissue. Receptors belonging to this family consist of an extracellular ligand-binding domain (ECD), a single transmembrane spanning region, an intracellular kinase homology domain and a GCase-catalytic domain. The binding of a specific ligand to the ECD activates intracellular GCase activity, resulting in conversion of GTP to cyclic 3′,5′-guanosine monophosphate (cGMP). Of the seven subtypes, GC-A is by far the best studied member; crystal structures are available for the ECD with and without the ligand [1,2]. GC-A and GC-B are also known as natriuretic peptide receptors (NPRs). The natriuretic peptide (NP) family consists of three structurally related hormones, namely, atrial NP (ANP), brain NP (BNP), and C-type NP (CNP). They are characterized by a disulfide-bonded loop of 17 highly conserved amino acid residues. ANP and BNP, with a C-terminal tail of five to six residues absent in CNP, bind to GC-A and play important roles in the regulation of diuresis, blood pressure, and water balance [3]. CNP binds specifically to GC-B, which mediates diverse biological activities with a widespread tissue distribution. The activities include endochondral ossification [4], relaxation of vascular smooth muscle cells [5,6], development of female reproductive organs [7], and antiproliferative and antihypertrophic actions [8,9].

In humans, reduction of GCase activity due to mutations in the *Npr2* gene that encodes GC-B causes autosomal recessive skeletal dysplasia [10,11]. CNP-knockout mice develop dwarfism due to impaired endochondral ossification [12]. CNP is now recognized as a novel drug for treatment of achondroplasia, the most common cause of human dwarfism [13], for which no effective cure was available. Over-expression or systemic administration of CNP rescued achondroplasia in the model mice, although the mechanism is unknown [14–16]. Thus, GC-B is clearly of pharmacological importance, but has been poorly characterized and never purified. In fact, presumably reflecting poor expression of GC-B, even the IC_{50} of CNP is not well determined. The reported values vary considerably from low picomolar to sub-nanomolar ranges [17–21], suggesting a much higher affinity compared to that of ANP binding to GC-A (~6.0 nM) [22].

As there is no naturally abundant source for NPRs, a prerequisite for characterization of GC-B is a method for large-scale production. Mammalian stable cell lines that secrete the ECD of GC-A have

Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; ECD, extracellular domain; FACS, fluorescence-activated cell sorting; GCase, guanylyl cyclase; GC-A, guanylyl cyclase-A receptor; GC-B, guanylyl cyclase-B receptor; HEK, human embryonic kidney; NP, natriuretic peptide.

* Corresponding author. Fax: +81 3 5841 8491.
E-mail address: ct@iam.u-tokyo.ac.jp (C. Toyoshima).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.077

been established [23] and led to the determination of its crystal structures [1]. Also, binding affinities of NPs were determined for GC-A and the ECD [22]. We intended to develop similar cell lines for both full-length and the ECD of GC-B. Here we demonstrate that it is indeed possible and even in a much shorter time by incorporating fluorescence activated-cell sorting (FACS) into the selection system. The system proves to be particularly efficient for the ECD, allowing us to purify it and characterize its properties accurately.

2. Materials and methods

2.1. Cell culture and transfections

HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 5–10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (Invitrogen, Grand Island, NY). The cells expressing the ECD were grown using roller bottles, with the culture medium renewed every 24 h. Transfections were performed by lipofection for stable expression; polyethyleneimine was used for transient expression [24]. For fluorescence microscopy, a Nikon ECLIPSE TE2000-U was used.

2.2. Stable expression of recombinant human full-length GC-B and the ECD

Highly expressing stable cell lines were established basically following the published protocol [25]. A cDNA encoding human GC-B (GenBank ID: NM_003995) was purchased from Origene (Rockville, MD). The coding sequences, including signal sequences, for the human full-length GC-B and the ECD (amino acid residues, 1–451) were amplified by PCR using the following three oligonucleotide primers: full-length and ECD sense: 5′-CGATCCCTCGAG GCTGCTTTATCCCCATGG-3′ (the restriction site is underlined); full-length antisense: 5′-CGATGCCCGCGGTTACAGGAGTCCAG-GAGG-3′; and ECD antisense: 5′-CGATGCCCGCGGCTAAGTTTTAT-CACAGGATGGG-3′. Each PCR product was subcloned into *Xho*I and *Sac*II sites of a cytomegalovirus promoter-driven pIRES2–AcGFP1 vector (Clontech, San Jose, CA). HEK293T cells were co-transfected with the construct and pPUR (Clontech) using Lipofectamine 2000 (Invitrogen). The transfected cells were selected in growth medium containing 0.2 μg/ml puromycin and cultured for 2 weeks. Then, the top 10% of the cell population with the highest fluorescence intensity was selected by GFP fluorescence on FACS Vantage SE cell sorter (Becton Dickinson, San Jose, CA). The selected cells were cultured with 0.2 μg/ml puromycin for one to 2 weeks, and subjected to another cycle of selection.

2.3. Sequencing of genomic DNA

Genomic DNA was extracted with FlexiGene DNA kit (Qiagen, Valencia, CA) from HEK293T cells expressing the full-length GC-B (FACS4) or the ECD (FACS5). The DNA encoding GC-B (or the ECD) was amplified by PCR with the following two oligonucleotide primers. Sense primer (5′-AGTGAACCGTCAGATCCGCT-3′) is located approximately 60 bp upstream from the full-length or the ECD coding region, and the sequence belongs to pIRES2–AcGFP1 vector. Antisense primer (5′-TCACTTGTACAGCTCATCCATGCC-3′) belongs to AcGFP coding sequence including the stop codon (underlined). Each PCR product was purified and sequenced to examine if any mutation was introduced into the GC-B gene incorporated.

2.4. Membrane preparation

Harvested HEK293T cells were suspended in a buffer consisting of 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 mM DTT, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, St. Louis, MO). The suspended cells were homogenized and centrifuged at 1220*g* for 30 min at 4 °C. The supernatant was further centrifuged at 200,000*g* for 1 h at 4 °C. The pellet was resuspended in a buffer containing PBS, pH 7.4, 1 mM EDTA, 0.5 mM DTT, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 mM PMSF, and stored at −80 °C until use. The protein concentration was determined with Pierce 660 nm Protein Assay Reagent.

2.5. Preparation of ligand-affinity columns and purification of the ECD

Two kinds of affinity gels were prepared using modified CNPs (Fig. 1A) synthesized by Takara Bio (Otsu, Japan) attached to Affi-Gel-10 (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The ECD of GC-B was affinity purified, following the protocol for ECD of GC-A [22]. Culture medium from the cells stably expressing the ECD was collected and filtered through a 0.22 μm Millipore HA filter. Protease inhibitors were added (final concentrations at 0.1 μg/ml aprotinin, 0.1 μg/ml leupeptin, 0.5 mM PMSF, and 1 mM EDTA) to the filtrate. The filtrate was applied to CNP–agarose columns equilibrated with equilibrium buffer consisting of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl. The columns were washed first with the equilibrium buffer, then a buffer consisting of 50 mM Tris–HCl, pH 7.4, 1 M NaCl, and finally with the equilibrium buffer again. The ECD was eluted with 100 mM sodium acetate, pH 5.0, and collected in 10 ml fractions in tubes containing 1 ml of 1 M HEPES–Na, pH 7.4, and 0.5 M NaCl for neutralization. The protein concentration was determined by the absorption at 280 nm.

2.6. Competitive binding assay with the purified ECD

The purified ECD was incubated with ^{125}I-[Tyr^{0}]CNP ([^{125}I]-CNP, Peninsula Lab., Merseyside, UK) (20,000 cpm per incubation) and varying concentrations of human NPs (Fig. 1B; Peptide Institute, Minoh, Japan) in PBS, pH 7.4, 0.05% (w/v) BSA (Sigma), and 0.01% (w/v) bacitracin (Fluka, Buchs, Switzerland) (PBS-BB). After incubation for 1 h at room temperature, [^{125}I]-CNP bound to the ECD was

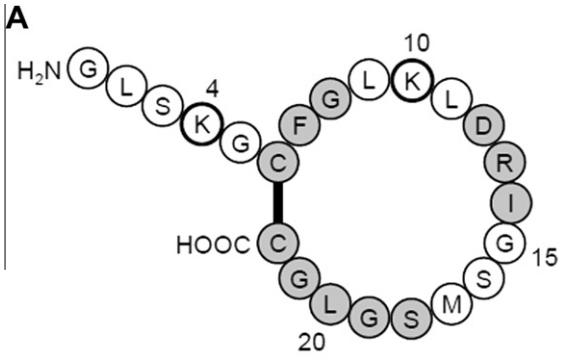

Fig. 1. Amino acid sequences of natriuretic peptides and the topology of the C-type one. (A) Topology of CNP. The disulfide bond and the substituted Lys's in the modified CNPs (bold circles) are marked. (B) Alignment of the human natriuretic peptides (NPs). ANP, atrial NP; BNP, brain NP; CNP, C-type NP. Conserved residues are shaded. Two Cys residues (bold) in each peptide form an intramolecular disulfide bond.

Download English Version:

https://daneshyari.com/en/article/10760643

Download Persian Version:

https://daneshyari.com/article/10760643

Daneshyari.com