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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

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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.

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.

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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

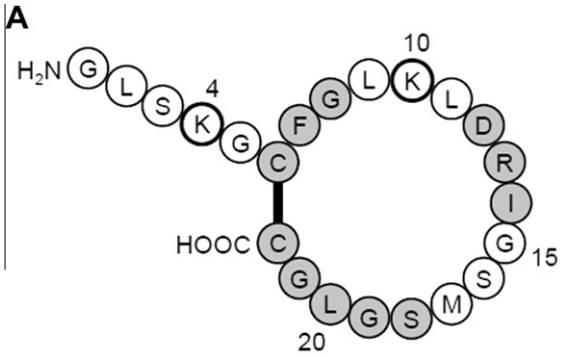

Fig. 1. Amino acid sequences of natriuretic peptides and the topology of the C-type one. (A) Topology of CNP. The disulfide bond and the substituted Lys's in the modified CNPs (bold circles) are marked. (B) Alignment of the human natriuretic peptides (NPs). ANP, atrial NP; BNP, brain NP; CNP, C-type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

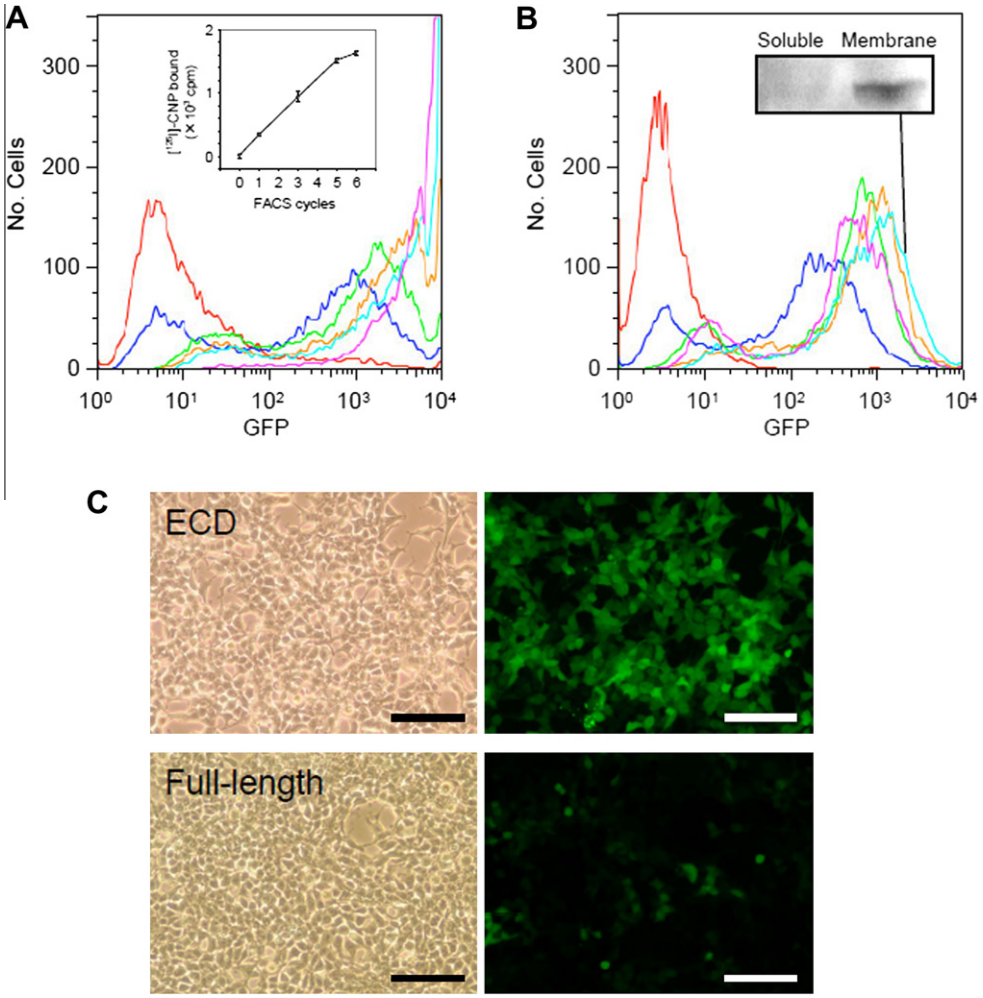

Fig. 2. FACS-based selection of high-expressing cells. FACS histogram overlays of cells expressing the full-length GC-B (B) and the ECD (A). Each histogram shows fluorescence intensity from AcGFP1 (*x*-axis; arbitrary scale) and numbers of cells (*y*-axis). Red, blue, green, yellow, cyan, and magenta lines represent histograms of cells before the first (FACS0), second (FACS1), third (FACS2), fourth (FACS3), fifth (FACS4), and sixth (FACS5) selections, respectively. Data were analyzed with FlowJo software (Tree Star, Ashland, OR). Variation in expression level of the ECD with FACS repeats quantitated in triplicate by the [^{125}I]-CNP binding assay with the culture medium is shown in (A). Western blot of a membrane preparation (60 μg of protein) from HEK293T cells stably expressing the full-length GC-B using an anti-GC-B ECD antibody is shown in (B). (C) Phase-contrast (left) and fluorescence microscopy (right) of HEK293T cells (FACS5) that express GC-B and the ECD. Scale bars represent 50 μm.

separated from free [^{125}I]-CNP by size-exclusion column chromatography and counted for ^{125}I-radioactivity in a gamma counter (COBRA 5010 Quantum; Packard, Meriden, CT). Non-specific binding was measured by competition with 1 μM unlabeled CNP and was 10–15% of the total binding.

2.7. Competitive binding assay with the full-length GC-B in membranes

The full-length GC-B in membranes was incubated with [^{125}I]-CNP and varying concentrations of NPs in PBS-BB for 1 h at room temperature, and filtered through Whatman GF/B glass fiber filters soaked in 0.3% polyethyleneimine to separate [^{125}I]-CNP bound to membrane from free [^{125}I]-CNP. After three washes of the filter, ^{125}I-radioactivity retained on the filter was determined by counting in the gamma counter. Non-specific binding was determined as described above.

2.8. cGMP assay

GCase activity was measured as described previously for GC-A [26]. Briefly, the full-length GC-B in membranes was incubated for 1 h at 37 °C in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM $MnCl_2$, 1 mM GTP, 0.1 mM ATP, 1 mM IBMX, 10 mM theophylline (Sigma), 1 mg/ml BSA, and varying concentrations of NPs. The reaction was stopped with 100 mM sodium acetate, pH 4.0, and 2 mM EDTA followed by incubation for 10 min at 100 °C. After centrifugation at 15,000*g* for 10 min at 4 °C, the supernatant was used for assay. The amount of cGMP produced was quantified using Cyclic GMP [^{3}H] Assay System (GE Healthcare). No NP-stimulated GCase activity was detected for endogenous NPRs in membranes prepared from mock-transfected cells.

3. Results

3.1. Establishment of HEK293T stable cell lines expressing the full-length GC-B and the ECD

To express the human full-length GC-B and the ECD, we used a bicistronic mammalian expression vector, pIRES2–AcGFP1, which contains the internal ribosome entry site and a sequence coding for a derivative of green fluorescent protein (AcGFP1), as the expression level of the target protein (i.e. full-length GC-B or the ECD) is expected to be proportional to that of AcGFP1. Monitoring

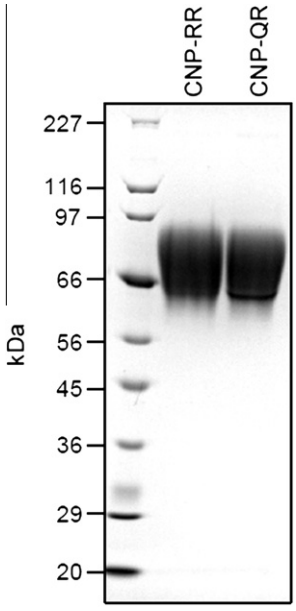

Fig. 3. Purification of the ECD by CNP-affinity chromatography. A CBB stained SDS-gel. Eight microgram of protein eluted from affinity-column using CNP-RR or CNP-QR was loaded. Molecular weight standards are also shown (lane 1).

fluorescence of the AcGFP1 allows selection of high-expressing cells (e.g. top 10%) by FACS. Selected cells are cultured further in the presence of 0.2 μg/ml puromycin and subjected to another cycle of selection. After several cycles of selection and culture, we expect that stable cell lines expressing the target proteins at very high levels have been established. This strategy worked well, in particular with the ECD, as shown in the FACS histograms (Fig. 2A).

The histograms show that the population of the cells with fluorescence levels higher than 10^4 (arbitrary scale) was insignificant initially (FACS0) but became prominent in the third to fifth cycles (FACS3–FACS5; Fig. 2A). Fluorescence microscopy of the cells in the fifth cycle (FACS5) confirmed that all the cells fluoresced very strongly and were morphologically uniform (Fig. 2C). The binding activity of the culture medium for [^{125}I]-CNP markedly increased, indicating that the ECD was secreted into the medium (Fig. 2A). The expression level of the ECD thus determined correlated well with the fluorescence from AcGFP1, and nearly reached a plateau at the fifth cycle (FACS5). We therefore used cells that went through six cycles of selection (FACS6) for purification and further characterization of the ECD.

The same strategy worked also with the full-length GC-B, but was not quite as successful as with the ECD. Initially (FACS0), the population of cells with a fluorescence level higher than 10^2 was negligible and the majority of cells had a level around 4. In FACS2–FACS5, the majority shifted to a fluorescence level of $\sim 10^3$ (Fig. 2B). However, the fluorescence declined after FACS4, suggesting that the expression level had reached its limit. Western blot of the membrane preparation from the cells in FACS4 showed a band corresponding to the expected size for the monomer (120 kDa) and reacted against an anti-GC-B ECD antibody. To compare the expression levels of the full-length GC-B in stable- and transient-expression, binding of [^{125}I]-CNP and CNP-dependent GCase activity were measured. The binding was ~1.5 times larger for membrane preparations from cells harvested at 36 h post-transfection than those from the stable cell line. The E_{max} of GCase activity was 20.2 ± 0.8 nmol/mg membrane protein/min with the membrane preparation from the stable cell line, and about equal to that of the preparation from cells transiently transfected (16.6 ± 2.9 nmol/mg membrane protein/min). Thus, the expression levels of GC-B in stable- and transient-expression are approximately the same.

Genomic DNA was extracted from the established cell lines, and the DNA encoding the full-length GC-B or the ECD was confirmed to have no mutation introduced. The binding activity of CNP for the ECD secreted into the culture medium and CNP-dependent GCase activity of the full-length GC-B in intact cells were monitored over five passages. The activities were constant within experimental error, showing that the established cell lines are stable and useful for further characterization.

3.2. Purification of the ECD by CNP-affinity chromatography

In the purification of the ECD of GC-A, affinity chromatography using ANP linked to a resin via the N-terminal amino group was very effective [22]. To apply a similar method to purify the ECD of GC-B, CNP has to be modified as it contains two Lys residues (Lys4 and Lys10; Fig. 1A). Affinity chromatography using the modified peptides, in which the two Lys residues were substituted with Arg-Arg (CNP-RR) or Gln-Arg (CNP-QR), was very efficient and yielded 1 mg of purified ECD of GC-B from 1 L of culture medium. This number compares favorably with that reported for the ECD of GC-A (0.1–0.4 mg/l of culture medium from transiently-expressing cells [22]). On SDS gels, the purified ECD appeared as a single broad

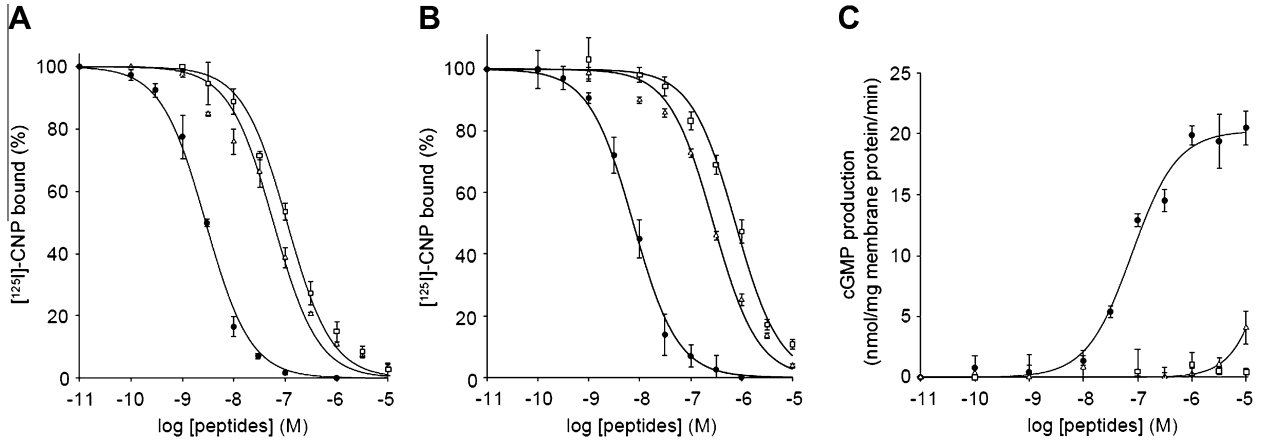

Fig. 4. Binding affinity of NPs for the full-length GC-B (A) and the purified ECD (B). (C) Activation of GCase activity of the full-length GC-B by NPs. ANP (open triangles), BNP (open squares) and CNP (filled circles) were used. Binding and dose–response data from triplicate experiments were analyzed with SIGMAPLOT 2000 (SPSS Inc, Chicago, IL). The curve fitting was performed by nonlinear regression based on the logistic equation to estimate IC_{50} or EC_{50}.

Table 1
IC_{50}'s and EC_{50}'s of various natriuretic peptides for GC-B measured with membrane preparations and the purified ECD.

Peptide	IC_{50} (nM)		EC_{50} (nM)
	ECD	Membranes	Membranes
ANP	280 ± 27	58.9 ± 9.5	ND
BNP	759 ± 63	109 ± 11	ND
CNP	7.5 ± 0.7	2.9 ± 0.5	78.2 ± 15.1
CNP-RR	60.0 ± 2.8	5.9 ± 0.8	
CNP-QR	162 ± 18	14.1 ± 1.2	

band of 60–90 kDa, which is much larger than the molecular mass expected from the amino acid sequence (48 kDa) (Fig. 3). This suggests that the ECD expressed in HEK293T cells is heavily and heterogeneously glycosylated.

3.3. Characterization of the full-length GC-B in membranes and of the purified ECD

To examine ligand selectivity, binding competition with [^{125}I]-CNP and ligand dependent GCase activity were measured for various NPs (Fig. 4) and listed in Table 1. The IC_{50} of CNP for the full-length GC-B in membranes is 2.9 ± 0.5 nM (Fig. 4A), and that for the ECD is 7.5 ± 0.7 nM (Fig. 4B). These values are substantially larger than those reported previously, as they range mostly from low picomolar to sub-nanomolar levels [17–21], yet are approximately the same as the IC_{50} of ANP for GC-A (3.1 nM with the full-length receptor and 6.0 nM with the ECD [22]). The selectivity ratio for CNP/ANP is ~20 with the full-length GC-B and ~40 with the ECD. These numbers appear not very large, compared to that of GC-A (>3000 for ANP/CNP [27]). However, ligand dependent GCase activity shows that the selectivity ratio is larger than 500 (Fig. 4C), indicating that CNP is, as expected, by far the most potent agonist of GC-B. Such difference in selectivity between GC-B and GC-A is understandable, because the central ring part of NPs is virtually identical and because the C-terminal tail of ANP that contributes to the binding to GC-A [1] is absent in CNP (Fig. 1B).

4. Discussion

In general, establishing cell lines that stably and highly express a recombinant protein is a time-consuming (usually >3 months) and laborious task. This is because, in addition to ordinary culturing in selective medium, it requires several cycles of dilution cloning, assessment of the expression level by, for instance, Western blotting and manual selection of high-expressing cells [21,28]. Our methods developed here have several advantages. The use of a bicistronic expression vector for co-expressing AcGFP1 makes it possible to assess the expression level of the target protein with individual cells and allows automatic and quantitative selection of high-expressing cells by FACS. As a result, establishing such cell lines took us less than 1.5 months for the full-length GC-B and the ECD. The purification system devised for the ECD was particularly effective, and provides us with 1 mg of purified protein per liter of culture medium. This amount is enough even for structural studies.

Our stable expression system for the full-length GC-B was of more limited success compared to that of the ECD, although the expression level was elevated >100-fold by FACS-based selection (Fig. 2B). The expression level (E_{max} of 20.2 nmol/mg membrane protein/min) achieved is similar to that in conventional transient-expression systems (23.5 nmol/mg membrane protein/min) [29]. Yet the system performs substantially better compared to previous stable cell lines (~1 to 5 nmol/mg membrane protein/min) [30,31] and is free from large variations in transfection efficiency. As stable cell lines are convenient for, amongst other uses, the assessment of various drugs, the system developed here may be particularly useful in characterizing other GCase receptors, for which physiological functions are yet to be identified, and in analyzing downstream signaling.

Acknowledgments

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