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Research paper

Expression, purification, and characterization of the intra-cellular domain of the ANP receptor

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ABSTRACT

The membrane-bound atrial natriuretic peptide receptor (GCA) catalyzes the formation of cGMP from GTP in response to natriuretic peptide hormones. Previous structural studies have focused on the extra-cellular hormone binding domain of this receptor whereas its intra-cellular domain has not yet been amenable to such studies. We report here the baculovirus expression and purification of the GCA intra-cellular domain construct GCA_{ID} comprising the complete intra-cellular region which includes the kinase-homology domain, coiled-coil region, and catalytic cyclase domain. The intra-cellular domain was enzymatically characterized in terms of guanylyl cyclase activity and the effects of ATP, manganese, and Triton X-100. Our results indicate that the activity of the intra-cellular domain of the ANP receptor is about 2 fold less active compared to a truncated cyclase domain construct lacking the kinase-like domain that was also expressed and purified. In addition, unlike the full length receptor, the intra-cellular domain could not be activated by Triton X-100/Mn^{2+} or its activity stimulated by ATP. These data therefore indicate that the major part of the transition from the basal state to the fully, ANP/ATP-dependent, activated state as well its stimulation/enhancement by Triton X-100/Mn^{2+} requires the full length receptor. These receptor insights could aid in the development of novel therapeutics as the GCA receptor is a key drug target for cardiovascular diseases.

1. Introduction

Guanylyl cyclases (GCs) are receptors that catalyze the formation of the second messenger cGMP from GTP in response to cellular signals. The atrial natriuretic peptide receptor A (GCA) is one of the best studied GCs and can be activated by atrial natriuretic peptide (ANP) or brain natriuretic peptide (BNP) hormones leading to vasodilation, diuresis and natriuresis among other effects with the net result of lowering blood pressure levels [1]. GCA is therefore a key drug target for treating a number of cardiovascular diseases such as congestive heart failure.

GCA can be topologically dissected into the following regions – the large extra-cellular ligand binding domain (ECD), a trans-membrane (TM) helix, an intra-cellular kinase-homology domain (KHD), a coiled-coil (CC) region, and the C-terminal guanylyl cyclase (GC) domain [2]. GCA is a pre-dimerized homo-dimer [3] with a strong dimerization role for the intra-cellular CC domain [4]; a homologous region in a related receptor was also found to be important for regulation/dimerization [5]. The crystal structure of the GCA ECD [6] and the hormone bound ECDs structure [7] provided structural insights into understanding of the role played by ECD in hormone recognition and relay of signaling information. However, structural insights into the intra-cellular domains (ID) are still lacking and how the ECD communicates with the GC domain in this process remains unknown. The KHD is situated centrally in between the signal-receiving ECD/TM half and the output catalytic CC/GC half of the GCA receptor and therefore likely plays a key role in the communication between the two halves of GCA.

In addition to the lack of intra-cellular domain structural insights, the precise role of the KHD and ATP is of debate with various, sometimes opposite, conclusions ranging from the KHD being inhibitory or regulatory, and ATP being either critical for activation or important for enzyme stabilization and/or sustaining activated receptor [8–12]. These studies are often done in cell lines with either transient or stably expressed receptor, assays done either in whole cells or with membrane preparation. While intra-cellular domain truncations of GCA have previously been expressed [21] and the catalytic domain of GCA has been purified and characterized [13], its full length intra-cellular domain has not yet been purified and characterized. To therefore investigate the role of GCA's KHD and ATP in a more isolated state, and also in preparation for structural studies, we over-expressed and purified the entire

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GCA intra-cellular domain. Although we realize the shortcomings of the divide-and-conquer method of not working with intact receptor, our ability to obtain purified intra-cellular domain allowed us to probe important questions regarding certain reported receptor enzymatic characteristics and ascribe these to either being inherent to the (isolated) intra-cellular domain, or to the full length receptor.

2. Materials and methods

2.1. Recombinant protein expression

A full length construct of GCA in vector pCMV3 was used for PCR amplification. GCA_{ID} was cloned into pFastbac-1 as a PCR product and corresponds to the entire intra-cellular GCA comprising amino acids 465–1029. Primers used: 5′cggggatccatgcatcatcatcatcatcatggt atgcagctggaaaaggagctggtc3′ and 5′gaagatcttcatcaacctcgagtgctacat cc3′ yielding a 6xHis-tag at the N-terminus; plasmid was sequenced to confirm the entire sequence. Recombinant bacmid DNA using DH10bac was generated (Bac-to-Bac, Invitrogen) and used to transfect Sf-9 cells using Cellfectin reagent to produce recombinant baculoviruses. For protein production, suspension cultures of High-Five were infected at a rate of 5–10 MOI at a cell density of 1.0–1.2 $\times$ 10^6 cells/ml. Cells were harvested after 72 h of infection. A shorter version without the KHD domain called GCA_{CC+GC}, comprising amino acids 776–1029, was generated using the primers 5′cacaggatccatatgaacagcagcaacatcctg3′ and 5′acagaattctcatcagcctcg agtgctaca3′ and subcloned into pET28a (Novagen) using restriction sites NdeI and EcoRI. The GCA_{CC+GC} containing fragment was subsequently subcloned into the pfastbac-1 vector using sites BamHI and EcoRI yielding a 6-His tag at the N-terminus followed by a thrombin cleavage site.

2.2. Protein purification

Cells containing recombinant protein were centrifuged and washed in saline phosphate buffer. Cell lysis was carried out by sonication in 50 mM sodium phosphate pH 8.0 containing 40 mM imidazole, 300 mM NaCl, 1 mM DTT, 10% glycerol, 5 μg/ml DNAseI, 10 μg/ml RNAse, and protease inhibitor cocktail (Sigma, USA). Following sonication, NP-40 was added to a final concentration of 1% and the lysates were incubated at 4 °C with gentle rocking for 1 h. Cell debris was removed by centrifugation at 30,000*g* for 30 min. Washed Ni-NTA beads (Qiagen, USA) were then added to the supernatants and incubated for 2 h at 4 °C while rocking. Beads were centrifuged, packed into a 50 ml column and washed 3$\times$ with lysis buffer with 1% NP-40 followed by 5$\times$ washing in large volumes (up to 15 times) of the same buffer without NP-40. Elution of the protein was carried out in a buffer containing 50 mM sodium phosphate pH 8.0, 250 mM imidazole, 300 mM NaCl, 1 mM DTT, 10% glycerol and the protease inhibitor cocktail. Immediately following elution, EDTA was added to the protein to a final concentration of 10 mM. The protein was subsequently buffer exchanged into 20 mM Tris–Cl pH 8.0, 1 mM DTT, and 10% glycerol prior to MonoQ anion exchange chromatography using a NaCl gradient. Final step of purification involved size-exclusion chromatography in a Superdex 200 10–30 column (GE health sciences) in a buffer containing 20 mM Tris–Cl, pH 8.0, 1 mM DTT, 150 mM NaCl and 10% glycerol. Protein concentrations were measured by Bradford method using BSA as a standard.

2.3. In vitro guanylyl cyclase assay

Assay for guanylyl cyclase activity was carried out in 50 mM Tris–Cl pH 7.4 containing 1 mM isobutyl methyl xanthine (IBMX), 5 mM creatine phosphate, and 20 μg/assay creatine phosphokinase, similar to [14]. Either $MgCl_2$ or $MnCl_2$ was used as metal cofactor. Unless otherwise mentioned, GTP concentration was maintained at 1 mM, $MgCl_2$ at 5 mM (or $MnCl_2$ at 2 mM). Reactions were carried out in 0.1 ml final volume at 37 °C for 10 min. After 10 min, 0.4 ml of 50 mM sodium acetate pH 4.5 was added to terminate the reaction followed by 3 min incubation in a boiling water bath. Samples were centrifuged before being aliquoted for cGMP measurements. Quantification of cGMP produced was carried out by a microtiter plate based immune adsorption method using the commercially available cGMP EIA kit (Cayman). Concentration of cGMP was measured against a standard curve of samples with known concentration. Each dilution was assayed in duplicate. A standard work sheet provided by the manufacturers was used for final calculation of cGMP concentration using Microsoft Excel.

2.4. Statistical analysis

GraphPad Prism (USA) was used for fitting enzyme kinetic curves. cGMP production as a function of substrate concentration was fitted to Hill equation:

$$v = \frac{V_{\max} S^{n_H}}{S_{0.5}^{n_H} + S^{n_H}}$$

where, $S_{0.5}$ is the K_m equivalent for cooperative enzyme kinetics, n_H is the Hill coefficient and V_{max} maximum enzyme velocity. For curves that obtained an n_H close to 1.0, a Michaelis–Menten equation was used for fitting.

3. Results and discussion

We have purified and enzymatically characterized the GCA_{ID} domain to allow comparison against results obtained from full length GCA receptors as well as against results from a published shorter intra-cellular domain construct comprised of only the catalytic region [13]. Such a truncated catalytic GCA construct, containing the CC + GC regions, was also expressed and purified in this study to allow comparisons under identical buffer conditions and to carry out additional experiments. Both constructs were expressed as N-terminal 6-His fusion proteins in High-Five cells and purified via affinity Ni-NTA chromatography followed by anion exchange and subsequent size-exclusion chromatography. Both GCA_{ID} and GCA_{CC+GC} eluted on the size-exclusion column close to that of their predicted dimeric state (data not shown). Fig. 1 confirms the purity and molecular weights of both proteins after they were purified. This purification involved cell culture volumes of around 5 L and resulted in a yield of around ~0.75 mg/L and ~0.3 mg/L for purified GCA_{CC+GC} and GCA_{ID} constructs, respectively. To ensure complete removal of the cell-lysis detergent NP-40, excessive washing of the Ni-NTA beads bound to the His-tagged proteins was carried out as well as the two subsequent chromatograph steps. The construct GCA_{CC+GC} contained a cleavable his-tag; its removal did not affect the gel elution profile nor activity (data not shown). On the other hand, the construct GCA_{ID} did not have a cleavable His-tag. We tried to excise the His-tag by treating with dipeptidyl aminopeptidase, DAPase (QIAGEN, USA). However, under our experimental conditions this reaction could not be reproducibly controlled. Due to the above reasons, and that His-tagged proteins often can be crystallized, further (enzymatic) studies were carried out with the His-tagged proteins.

All the guanylyl cyclases studied so far have shown considerably higher catalytic activity in the presence of Mn^{2+} ions compared to Mg^{2+}. To systematically compare the effects of these ions, we measured guanylyl cyclase activity at increasing concentrations of