Journal of Structural Biology 172 (2010) 94–106

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Regular Article

Modulation of G-protein coupled receptor sample quality by modified cell-free expression protocols: A case study of the human endothelin A receptor

Friederike Junge [a], Laura M. Luh [a], Davide Proverbio [a], Birgit Schäfer [a], Rupert Abele [b], Michael Beyermann [c], Volker Dötsch [a], Frank Bernhard [a,*]

[a] Centre for Biomolecular Magnetic Resonance, Institute for Biophysical Chemistry, Goethe-University of Frankfurt/Main, Max-von-Laue-Str. 9, D-60438 Frankfurt/Main, Germany
[b] Institute of Biochemistry, Goethe-University of Frankfurt/Main, Max-von-Laue-Str. 9, D-60438 Frankfurt/Main, Germany
[c] Leibniz-Institute of Molecular Pharmacology (FMP), Robert-Rössle Str. 10, 13125 Berlin, Germany

ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 3 May 2010
Accepted 5 May 2010
Available online 9 May 2010

Keywords:
Cell-free expression
Endothelin A receptor
G-protein coupled receptors
Solubilization
Ligand affinity chromatography
Agonist binding

ABSTRACT

G-protein coupled receptors still represent one of the most challenging targets in membrane protein research. Here we present a strategic approach for the cell-free synthesis of these complex membrane proteins exemplified by the preparative scale production of the human endothelin A receptor. The versatility of the cell-free expression system was used to modulate sample quality by alteration of detergents hence presenting different solubilization environments to the synthesized protein at different stages of the production process. Sample properties after co-translational and post-translational solubilization have been analysed by evaluation of homogeneity, protein stability and receptor ligand binding competence. This is a first quality evaluation of a membrane protein obtained in two different cell-free expression modes and we demonstrate that both can be used for the production of ligand-binding competent endothelin A receptor in quantities sufficient for structural approaches. The presented strategy of cell-free expression protocol development could serve as basic guideline for the production of related receptors in similar systems.

1. Introduction

Efficient production of high quality samples is a major pacemaker in structural approaches of membrane proteins (MPs). Their hydrophobic nature, toxic effects and specific requirements for targeting and translocation systems still make them to one of the most difficult class of proteins (Drew et al., 2003; Dalbey and Chen, 2004; Wagner et al., 2006). Particularly in *Escherichia coli*, the most commonly used heterologous expression host, overexpression of MPs can result into the accumulation of aggregated material. While refolding of these inclusion bodies can be successful for β-barrel MPs, it so far failed in most cases for the far more abundant MPs characterized by an α-helical topology. Preparative scale cell-free (CF) expression systems have become established tools for the production of diverse kinds of proteins (Yokoyama, 2003; Spirin, 2004; Endo and Sawasaki, 2005; Junge et al., 2008; Katzen et al., 2009). CF expression reduces the high complexity of protein production known from living organisms to the basic translation process. Most toxic effects of synthesized proteins to the expression host are virtually eliminated and the speediness of CF expression is highly competitive as reactions are usually finished within 10–20 h. Those features render CF approaches often more reliable and reproducible if compared with many cell-based expression systems. The most powerful characteristic of CF systems is their open nature that allows the addition of compounds at any time point of the reaction. Freshly translated proteins can thus instantly be stabilized by diverse arrays of supplemented additives, such as ligands, co-factors, inhibitors or specific lipids.

CF systems have opened completely new ways to synthesize MPs and currently three different general expression modes can

Abbreviations: B35, Brij-35; B58, Brij-58; B78, Brij-78; B98, Brij-98; CD, circular dichroism; CF, cell-free; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propansulphonat; CHS, cholesteryl hemisuccinate tris salt; CMC, critical micellar concentration; CV, column volume; D-CF, detergent based cell-free; DDM, *n*-dodecyl-β-D-maltoside; ET-1, endothelin-1; ETA, endothelin A receptor; ETB, endothelin B receptor; FM, feeding mixture; Fos-12, *n*-dodecylphosphocholine; Fos-16, *n*-hexadecylphosphocholine; GPCR, G-protein coupled receptor; HDM, *n*-hexadecyl-β-D-maltopyranoside; IC_{50}, 50% inhibitory concentration; IMAC, immobilized metal affinity chromatography; kDa, 10^3 Dalton; L-CF, lipid based cell-free; LMPC, 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac(1-choline)]; LMPG, 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; LPPG, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; MALS, multi angle light scattering; MP, membrane protein; MW, molecular weight; NMR, nuclear magnetic resonance; PEI, polyethyleneimine; P-CF, precipitate generating cell-free; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RM, reaction mixture; SEC, size exclusion chromatography; SDS, sodium dodecylsulphate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; TDM, *n*-tetradecyl-β-D-maltopyranoside; TMS, transmembrane segment; TX-100, Triton X-100.

* Corresponding author. Fax: +49 69 798 29632.
E-mail address: fbern@bpc.uni-frankfurt.de (F. Bernhard).

1047-8477/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2010.05.004

be distinguished (Junge et al., 2008; Schwarz et al., 2007). In the P-CF (precipitate generating) mode, no hydrophobic compounds are added into the reaction and translated hydrophobic MPs will consequently form a precipitate. This process apparently resembles the inclusion body formation in *E. coli* cells. However, increasing numbers of reports already indicate that even complex MPs can be functionally reconstituted from CF precipitates (Klammt et al., 2004; Ishihara et al., 2005; Kamonchanok et al., 2008; Keller et al., 2008). In contrast to the solubilization of inclusion bodies, extensive refolding protocols including high concentrations of chaotropic agents need not to be applied. However, more detailed structural comparisons of inclusion bodies and P-CF produced precipitates supporting this different behaviour are still missing. Supplementation of detergents in the D-CF (detergent based) mode enables the instant solubilization of MPs during or shortly after translation. This unique MP production mode generates proteomicelles directly in the expression environment which stay in the soluble part of the reaction. In the L-CF (lipid based) mode, predominantly defined lipid bilayers are provided for the stabilization of MPs (Kalmbach et al., 2007; Gourdon et al., 2008; Katzen et al., 2008; Wuu and Swartz, 2008). However, efficient translocation can become limiting again in the L-CF mode and most of the synthesized MPs might still precipitate.

G-protein coupled receptors (GPCRs) are the most abundant class of MPs encoded by the human genome and are the key players in signal perception and transduction (Kristiansen, 2004; Rosenbaum et al., 2009). The central signature motif of the GPCR superfamily is a seven transmembrane segment (TMS) topology confined by an N-terminal and a C-terminal soluble domain. Ligand binding to GPCRs induces downstream signalling pathways triggered through dynamic interaction with heterotrimeric G-protein complexes. The cytoplasmic C-terminal domain of GPCRs has often been identified to be crucial for G-protein coupling. The eminent role of GPCRs in sensing environmental signals makes them consequently to one of the currently most important class of drug targets (Klabunde and Hessler, 2002). Heterologous overexpression as an indispensable prerequisite for structural analysis of most GPCRs has been a challenge now for decades and conventional cell based systems have extensively been analysed (Sarramegna et al., 2003; Tate et al., 2003; Grisshammer et al., 2005; Lundstrom, 2006). In particular encouraging are few recently successful structural approaches, although resulting from sophisticated and elaborated strategies (Cherezov et al., 2007; Rasmussen et al., 2007; Warne et al., 2008).

The human endothelin system comprises the two class A GPCR subtypes endothelin receptor A (ETA) and endothelin receptor B (ETB). Both receptors are primarily located in vascular smooth muscles and in the endothelium (Davenport, 2002). Despite 59% amino acid identity, the signalling function and targeting mechanisms of the activated ETA and ETB receptors are quite different (Sakurai et al., 1992; Cramer et al., 2001). Vasoconstriction and cardiac inotropy is a result of ETA stimulation whereas vasodilatation can be attributed to ETB (Haynes and Webb, 1998). The endothelin system generally is a major modulator in cardiovascular regulation and shows a striking diversity in biological responses affecting vasoconstriction, neurotransmission, embryonic development, mitogenesis, renal function, hormone production and even cancer. In many corresponding pathophysiological conditions like chronic heart failure or pulmonary hypertension, endothelin antagonism could therefore be a possible clinical treatment (Bagnato and Natali, 2004). Identified natural agonists of the endothelin receptors are the three 21-amino acid isopeptides ET-1, ET-2 and ET-3. The principal natural agonist in the human cardiovascular system is ET-1, which displays equal affinities to the ETA and ETB receptors (Russell and Molenaar, 2000).

We have selected the ETA receptor as a model target for evaluating CF expression strategies as a preparative scale production pipeline of GPCRs. In previous work, the CF production of few other GPCRs including human ETB have been reported (Ishihara et al., 2005; Kamonchanok et al., 2008; Kaiser et al., 2008; Klammt et al., 2007). However, different CF systems have been used and only limited systematic analysis of different expression modes or detergents has been done. The ETA receptor has been produced in the P-CF and D-CF mode and effects of a variety of different detergents on sample homogeneity, stability and ligand binding were analysed. We demonstrate that the ETA receptor can be produced with variety of CF protocols, resulting in qualities apparently sufficient for further functional and structural approaches.

2. Materials and methods

2.1. Materials

Wild type ET-1 and its biotinylated derivative Lys^9 – biotin – ET-1 were obtained from the Leibniz Institute for Molecular Pharmacology, FMP, Berlin, Germany). Biotinylated (Arg^8) – vasopressin trifluoracetate salt was purchased from BACHEM (Weil am Rhein, Germany) and the fluorescein labelled 4-alanine 1,3,11,15 mutant ET-1 (f-4-Ala-ET-1) was synthesized by BIOSYNTAN (Berlin, Germany). The radioactively labelled ET-1 (Endothelin-1 (Human, Porcine), [^{125}I] Tyr^{13}-ET-1, 2200 Ci/mmol) was purchased from PerkinElmer (Rodgau-Jügesheim, Germany).

2.2. DNA techniques

The coding region of the human ETA receptor was PCR-amplified from cDNA obtained from the UMR cDNA Resource Center (www.cdna.org) by using VentDNA-polymerase (New England Biolabs, Frankfurt Germany) and the oligonucleotide primers ETAforward: 5′-CGAAGATCTATGGAAACCCTTTGCCTCAGGGCATCC-3′ and ETAreverse: 5′-CCGCTCGAGCATGCTGTCCTTATGGCTGCTCCG-3′. Restriction sites for the enzymes BglII and XhoI were introduced by suitable linkers. The coding region of the human ETB receptor without its signal peptide was PCR-amplified using a full-length receptor containing plasmid as template (Klammt et al., 2007) and the oligonucleotide primers ETBforward: 5′-CGGGGATCCGAGGAA GAGGCTTCCCGCCTGACAGG-3′ and ETBreverse: 5′-CGGCTCGAGAG ATGAGCTGTATTTATTACTGGAACG-3′. Restriction sites for the enzymes BamHI and XhoI were introduced by appropriate linkers. PCR fragments were purified, restriction digested and ligated with a BamHI and XhoI digested derivative of the vector pET21a(+) (Merck, Darmstadt, Germany) encoding for a poly$(His)_{10}$-tag. ETA and ETB were expressed from these constructs with a N-terminal T7-tag and a C-terminal poly$(His)_{10}$-tag. Plasmid DNA used as template for CF expression was isolated with commercial kits (Macherey–Nagel, Düren, Germany) according to manufacturer's recommendations.

2.3. Cell-free expression

Basic CF expression protocols were previously described (Schwarz et al., 2007; Klammt et al., 2005). Bacterial S30 extract were prepared from *E. coli* strain A19 and T7 RNA polymerase was overexpressed in BL21 (DE3) Star cells and purified as described (Schwarz et al., 2007). Analytical scale reactions for the optimization of expression conditions were performed in 24-well microplates in Mini-CECF reactors (Kopeina et al., 2008; Schneider et al., 2010). Appropriate dialysis membranes, type 27/32, having a molecular weight cut-off of 12–14 kDa (Roth, Karlsruhe, Germany) were used to separate reaction mixture (RM) from feeding mixture (FM). The RM volume was 55 μl with a RM to FM ratio of 1:15. Preparative scale expression was accomplished in Maxi-CECF reactors (Schneider