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Co-translational formation and pharmacological characterization of beta1-adrenergic receptor/nanodisc complexes with different lipid environments

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ABSTRACT

G protein-coupled receptors are of key significance for biomedical research. Streamlined approaches for their efficient recombinant production are of pivotal interest in order to explore their intrinsic conformational dynamics and complex ligand binding behavior. We have systematically optimized the co-translational association and folding of G protein-coupled receptors with defined membranes of nanodiscs by cell-free expression approaches. Each optimization step was quantified and the ligand binding active fraction of the receptor samples could drastically be improved. The strategy was exemplified with a stabilized and a non-stabilized derivative of the turkey beta1-adrenergic receptor. Systematic lipid screens with preformed nanodiscs revealed that generation of ligand binding active conformations of the analyzed beta1-adrenergic receptors strongly depends on lipid charge, flexibility and chain length. The lipid composition of the nanodisc membranes modulates the affinities to a variety of ligands of both receptor derivatives. In addition, the thermostabilization procedure had a significant impact on specific ligand affinities of the receptor and abolished or reduced the binding of certain antagonists. Both receptors were highly stable after purification with optimized nanodisc membranes. The procedure avoids any detergent contact of the receptors and sample production takes less than two days. Moreover, even non-stabilized receptors can be analyzed and their prior purification is not necessary for the formation of nanodisc complexes. The established process appears therefore to be suitable as a new platform for the functional or even structural characterization of recombinant G protein-coupled receptors associated with defined lipid environments.

1. Introduction

The abundance of more than 800 different G protein-coupled receptors (GPCRs) encoded by human cells indicates their key position in signal perception and overall cell physiology as well as their important role in biomedical research [1]. Structural studies, directed engineering and extended ligand screening of GPCRs commonly require their overproduction in heterologous expression systems. Intensive research during the past decades resulted into elaborated strategies for the production and *in vivo* characterization of GPCRs in particular by implementing mammalian or insect cell cultures [1,2]. However, the isolation and *in vitro* characterization of GPCRs still pose significant problems that are mainly based on their intrinsic instability, their high conformational dynamics and their frequent sensitivity against standard isolation procedures involving detergents.

Cell-free (CF) expression systems based on lysates of *Escherichia coli* (*E. coli*) cells have emerged as efficient and highly versatile production platforms in particular for membrane proteins [3]. Central bottlenecks known from conventional cell-based membrane protein production are removed by the reduced complexity of CF systems and by a large diversity of options to co-translationally stabilize membrane proteins

Abbreviations: GPCR, G protein-coupled receptor; CF, cell-free; β1AR, beta1-adrenergic receptor; Brij35, polyoxyethylene-(23)-lauryl-ether; Brij58, polyoxyethylene-(20)-cetyl-ether; Brij78, polyoxyethylene-(20)-stearyl-ether; CHS, cholesteryl-hemisuccinate; LMPG, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phospho-rac-(1-glycerol); Fos-16, fos-choline 16; DPC, dodecyl-phosphocholine; DDM, n-dodecyl-D-maltoside; SDS, sodium-dodecyl-sulfate; DH_7PC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol); POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; SOPG, 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DOPA, 1,2-dioleoyl-sn-glycero-3-phosphate; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; DEPG, 1,2-dielaidoyl-sn-glycero-3-phospho-(1′-rac-glycerol); Aso-PC, L-α-phosphatidylcholine from soybean, type IV-S; ETL, *Escherichia coli* total lipid extract; HTL, heart total lipid extract; sfGFP, superfolder green fluorescent protein; ND, nanodisc; SEC, size exclusion chromatography; CECF, continuous exchange cell-free expression; RM, reaction mixture; FM, feeding mixture; P-CF, precipitate forming cell-free expression; D-CF, detergent-based cell-free expression; L-CF, lipid-based cell-free expression; IMAC, immobilized metal-chelate affinity chromatography; CV, column volume; LAC, ligand-affinity chromatography; ^{3}H-DHA, [^{3}H]dihydroalprenolol.

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and to solubilize them in a variety of artificial hydrophobic environments [4,5]. Applications of CF synthesized membrane proteins are continuously expanding and already include crystallization [6,7], structural analysis by NMR spectroscopy [8,9] and even studies of large membrane protein assemblies by electron microscopy [10].

Due to their importance, GPCRs represent one of the most prevalent types of membrane proteins studied so far by CF expression. Numerous members of the most abundant class A or rhodopsin-type GPCRs have been CF expressed and specific ligand binding activities have been shown [11–14]. The CF production efficiency of GPCRs is usually remarkable and even mg amounts can be obtained out of one ml of CF reaction [4]. However, data on stability, specific activities or percentages of ligand binding fractions in CF synthesized GPCR samples are still rare. Only less than 1% of CF synthesized human endothelin B receptor was found to be ligand binding active, regardless whether it was expressed in either *E. coli* lysates [13] or insect cell lysates [15].

The high demand for molecular structural details of GPCRs requests the development of procedures for the generation of homogenous and stable samples with high specific activities. In particular the thermostabilization of GPCRs by directed engineering resulted into several high resolution structures [16]. Derivatives of the turkey beta1-adrenergic receptor (β1AR) are amongst the best studied examples by this strategy [17–20].

In this work we identify parameters that considerably improve the specific activity of CF synthesized GPCRs exemplified with the β1AR. A thermostabilized as well as a non-stabilized β1AR derivative were CF synthesized in the presence of a variety of artificial hydrophobic environments and analyzed by quantitative ligand binding assays. A systematic lipid screening with preformed nanodiscs revealed a strong correlation of β1AR functional conformations to particular lipid features. We furthermore demonstrate the modulation of β1AR ligand affinities by different lipid environments. In addition, the two analyzed β1AR derivatives show differential ligand binding characteristics that can be attributed to the individual modifications in their primary structure. The reported strategy appears therefore to be suitable for the rapid mapping of GPCR ligand binding properties solubilized with defined lipid environments.

2. Materials and methods

2.1. Detergents, lipids and ligands

Polyoxyethylene-(23)-lauryl-ether (Brij35), polyoxyethylene-(20)-cetyl-ether (Brij58), polyoxy-ethylene-(20)-stearyl-ether (Brij78), cholesteryl-hemisuccinate (CHS) and digitonin were obtained from Sigma-Aldrich (Taufkirchen, Germany). 1-Myristoyl-2-hydroxy-*sn*-glycero-3-phospho-rac-(1-glycerol) (LMPG) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Fos-choline 16 (Fos-16), dodecylphosphocholine (DPC) and *n*-dodecyl-D-maltoside (DDM) were obtained from Anatrace (High Wycombe, UK). Sodium-dodecyl-sulfate (SDS) was obtained from Roth (Karlsruhe, Germany).

1,2-Diheptanoyl-*sn*-glycero-3-phosphocholine (DH_7PC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (SOPG), 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dielaidoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DEPG), L-α-phosphatidylcholine from soybean, type IV-S (Aso-PC), *E. coli* total lipid extract (ETL) and bovine heart total lipid extract (HTL) were purchased from Avanti Polar Lipids (Alabaster, USA). Alprenolol and carvedilol were purchased from Tocris Bioscience (Bristol, UK). Labetalol, metoprolol and nebivolol were purchased from Sigma Aldrich (Steinheim, Germany). All other chemicals were purchased from Roth (Karlsruhe, Germany) if not mentioned elsewhere.

Stock solutions of detergents were prepared with a concentration of 5% (w/v) for Brij derivatives, 8% (w/v) for digitonin and 10% (w/v) for LMPG, DPC and DDM with water as solvent. Lipid or detergent stocks were prepared by measuring appropriate amounts of substances into a vial. Stocks containing 50 mM lipid or 300 mM sodium cholate were prepared in water. Solubilization of the substances was supported by vortexing and sonication in an ultrasonic bath at 37 °C until solutions became clear. Lipid and detergent stock solutions were stored at −20 °C.

2.2. DNA and protein techniques

A truncated version of turkey β1AR (Δβ1AR) and its thermostabilized derivative tsΔβ1AR were analyzed by CF expression. Δβ1AR contains deletions of the N- and C-terminal domains from amino acid positions 1–43 and 368–483 and in addition the point mutation C116L. The thermostabilized derivative tsΔβ1AR is truncated at the N-terminal domain at amino acid positions 3–32 and contains the same C-terminal deletion like Δβ1AR from amino acid position 368–483. It further contains the point mutations C116L, R68S, M89V, I129V, E130W, Y227A, A282L, F327A, F338M, Y343L and C358A in order to increase thermostability. Furthermore, part of the intracellular loop 3 from position 244–271 is deleted.

DNA templates of tsΔβ1AR-sfGFP-H6, tsΔβ1AR-H10 and Δβ1AR-sfGFP-H6 in pET21a(+) expression vector were kindly provided by Ali Jazayeri (Heptares Therapeutics, Welwyn Garden City, UK). The Δβ1AR protein was modified with a C-terminal superfolder green fluorescent protein (sfGFP) tag and a C-terminal poly$(His)_6$-tag resulting into the Δβ1AR-sfGFP-H6 construct. The tsΔβ1AR protein was modified either with a C-terminal poly$(His)_{10}$-tag (tsΔβ1AR-H10) or a C-terminal sfGFP-tag and a C-terminal poly$(His)_6$-tag (tsΔβ1AR-sfGFP-H6). The coding regions of all three constructs were synthesized and cloned into the vector pET21a(+) and expressed under control of the T7 promoter.

Protein samples were analyzed by SDS-PAGE and western blotting as described previously [13]. Protein staining in SDS-gels was done by incorporation of 2,2,2-trichloroethanol [21]. Total protein concentration in the samples was determined by the Bradford-assay or by absorbance at 280 nm. Target protein concentration was monitored by fluorescence of the C-terminal sfGFP fusion and quantified on a TECAN Genius Pro fluorescence reader (Tecan, Männedorf/Zürich, Switzerland) as described earlier [22].

2.3. Nanodisc preparation

Nanodisc (ND) preparation was done as described earlier [23,24]. Briefly, the scaffold protein MSP1E3D1 was expressed in *E. coli* strain BL21 Star (DE3) for 1 h at 37 °C followed by 4 h at 28 °C and purified via IMAC affinity chromatography. MSP1E3D1 protein and lipids were mixed together with 0.1% (w/v) DPC for 1 h at room temperature under gentle stirring. Molar MSP1E3D1 to lipid ratios were taken from reference [24]: 1:115 (DMPC), 1:110 (DMPG), 1:85 (POPC), 1:80 (DOPC), 1:80 (DOPE). For POPG (1:90) and DOPG (1:80) the MSP1E3D1 to lipid ratios were slightly adjusted if compared with reference [23]. For other lipids, suitable molar MSP1E3D1 to lipid ratios were determined by size exclusion chromatography (SEC) profiling of a series of samples assembled with varying ratios as described in reference [24]. The ratios giving the apparently most homogeneous SEC profiles were: 1:90 (POPS), 1:80 (SOPG), 1:90 (DOPA), 1:90 (DOPS), 1:80 (DEPG), 1:50 (Aso-PC), 1:50 (ETL), and 1:50 (HTL). Preparations were dialyzed for 48 h at room temperature against 500-fold excess of ND formation buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl) with buffer exchange after 10 h and 24 h. Aggregates were removed by centrifugation for