Polymer-based cell-free expression of ligand-binding family B G-protein coupled receptors without detergents

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Abstract: G-protein coupled receptors (GPCRs) constitute the largest family of intercellular signaling molecules and are estimated to be the target of more than 50% of all modern drugs. As with most integral membrane proteins (IMPs), a major bottleneck in the structural and biochemical analysis of GPCRs is their expression by conventional expression systems. Cell-free (CF) expression provides a relatively new and powerful tool for obtaining preparative amounts of IMPs. However, in the case of GPCRs, insufficient homogeneity of the targeted protein is a problem as the *in vitro* expression is mainly done with detergents, in which aggregation and solubilization difficulties, as well as problems with proper folding of hydrophilic domains, are common. Here, we report that using CF expression with the help of a fructose-based polymer, NV10 polymer (NVoy), we obtained preparative amounts of homogeneous GPCRs from the three GPCR families. We demonstrate that two GPCR B family members, corticotrophin-releasing factor receptors 1 and 2β are not only solubilized in NVoy but also have functional ligand-binding characteristics with

different agonists and antagonists in a detergent-free environment as well. Our findings open new possibilities for functional and structural studies of GPCRs and IMPs in general.

Introduction

Integral membrane proteins (IMPs), situated at the cell surface, are often the mediators of intercellular communication and transmembrane (TM) transport of substances and signals between the outside and the inside of the cell. Among all IMPs, G-protein coupled receptors (GPCRs) are the largest class of cell-surface receptors which, according to the GPCR database, are encoded by more than 1000 genes in the human genome.[1] Many physiological regulatory mechanisms in the human body are controlled by GPCRs, and it is estimated that about 50% of all modern drugs target this family.[2] The GPCR sequences are divided into three major families: the largest subgroup, family A, also referred to as the rhodopsin-like family, comprises receptors for odorants, small molecules, and peptides and glycoprotein hormones; family B is characterized by a relatively long amino terminus that contains several disulfide-linked cysteines; family C is characterized by a long amino terminus and a unique short and highly conserved third intracellular loop.

To obtain preparative amounts of GPCRs, heterologous cell-based expression using *E. coli*, yeast, insect, or mammalian cells have been widely optimized.[3–7] However, the successful synthesis in cellular systems is still limited and preparative amounts of GPCRs are in most cases beyond reach, although a few notable successes in GPCR structure determination have been achieved, including the highly abundant bovine rhodopsin,[8] human β2-adrenergic receptor expressed in insect cells,[9,10] and crystallized with monoclonal antibodies[11] or expressed as T4-lysozyme fusion to aid crystallization,[12] human A2A adenosine receptor,[13] and turkey β1-adrenergic receptor.[14]

E. coli-based cell-free (CF) expression systems have been optimized for large scale expression of IMPs,[15–18] and up to 6 mg GPCR per mL of CF reaction mixture (RM) has been produced in the individual continuous exchange CF (CECF) system.[19] The open nature of CF systems allows the expression of IMPs in two basically different modes. In mode 1, insoluble cell-free expression as precipitate (P-CF), in the absence of any hydrophobic environment, IMPs are CF-produced as precipitate, which can be subsequently solubilized in mild detergents.[17,20] In mode 2, soluble CF expression (S-CF), the addition of certain detergents (S_D-CF) or lipids (S_L-CF) not interfering with the protein expression machinery allows a direct soluble expression of IMPs into detergent micelles[19,21] or reconstitution into lipid molecules[22,23] or nanolipoprotein particles.[24] Preparative scale CF production of diverse GPCRs by these methods[19–21] has enabled a significant functional characterization of some members of the A GPCR family.[21,25–29]

Successful solubilization of IMPs preserving their native conformation is another prerequisite for functional and structural analyses. However, nonspecific aggregation of detergent-solubilized proteins is a common problem.[30] Another major problem is related to the balance between the concentration of detergent needed to keep the hydrophobic TM domain of the protein soluble and the concentration required to keep its hydrophilic domains properly folded. To resolve these problems, extensive screening for optimal buffer and detergent conditions for homogenous GPCR samples is often carried out.[27,31] Another solution has been to bypass the use of detergents. Tribet *et al.*, for example, introduced amphipols (APs), amphiphilic polyacrylate-derived polymers grafted with hydrophobic chains,[32] which helped keep IMPs in their active form.[33,34] Although APs keep IMPs soluble in the absence of detergent for a wide range of proteins,[35] charged AP molecules can cause the same problems in CF systems as those found when charged detergents are used.[19]

In this article, we demonstrate that CF expression of various GPCRs from the three major families can be achieved with a polyfructose-based uncharged NV10 polymer (Expedeon Protein Solutions, UK) (NVoy) (www.expedeon.com). GPCRs CF-expressed in the presence of NVoy are homogeneous in solution, compactly folded, and yet show specific ligand binding to their extracellular domains. We characterize NVoy-solubilized corticotrophin-releasing factor receptors 1 (CRFR1) and 2β (CRFR2β), both family B GPCRs, for their ligand-binding specificity and affinity in the absence of detergents.

Results

GPCRs were S-CF expressed in the CF expression system in the presence of NVoy (S_N-CF)

After establishing NVoy suitability for membrane protein studies (see Supporting Information and Supporting Information Fig. S1), we tested it for a possible interference with the CF expression machinery. We analyzed the expression of the reporter protein GFP in the presence and absence of the
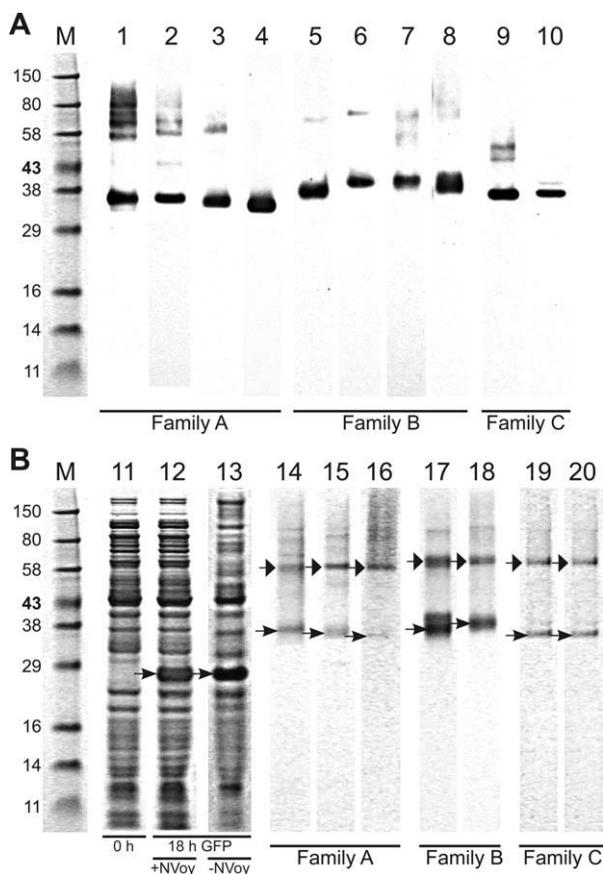
Figure 1. CF expression of GPCRs of all three sub-families. A: Western blot verification of solubilized and purified GPCRs by Anti-His$_6$ antibody (lane 1, 2, 5, 6) or Anti T7 tag antibody (lane 3, 4, 7–10). GPCRs CCR1 (1), CCR5 (2), SSR2 (3), SSR5 (4), CRFR2β (7), GPCRC5b (9), and RAI3 (10) were obtained from S$_N$-CF; CRFR1 (5) and CRFR2β from P$_N$-CF (6); and CRFR2β from P$_L$-CF (8). B: Coomassie-stained analysis of the soluble fraction of the CF reaction mixture in presence of 0.5 mM NVoy before (11) and after 18 h of GFP expression (12); of 18 h GFP expression in absence of NVoy (13); and of purified GPCRs. CCR1 (14), CCR5 (15) SSR2 (16), CRFR1 (17), CRFR2β (18), GPRC5b (19), and RAI3 (20) where obtained from P$_N$-CF. The corresponding GPCR family type is indicated below the lanes. CF-expressed proteins, running as monomers and dimers on SDS-PAGE, are marked with arrows.

polymer. In both cases, with 0.5 mM NVoy (Fig. 1, lane 12) and without NVoy (Fig. 1, lane 13), GFP expressed up to 3 mg/mL in the individual CF system. Following these results, we analyzed the S-CF expression of various GPCRs in the presence of NVoy. Using NVoy at 0.5 mM in the RM enabled soluble expression of GPCRs from the three major GPCR families (Table I). All tested GPCRs were purified by immobilized metal ion affinity chromatography (IMAC) against a C-terminal His$_6$-tag in the presence of NVoy but no detergent, and verified by Western blot using antibodies against the His6 or an optional N-terminal T7 tag [Fig. 1(A)]. It is noteworthy that the apparent sizes of expressed GPCRs

on SDS-PAGE are smaller than their actual size due to anomalous SDS-PAGE migration of membrane proteins.[36]

To compare S$_N$-CF with CF expression in the presence of APs, the commercially available AP polymer PMAL-B-100 was tested. PMAL-B-100 was found to inhibit the GFP expression at concentrations above 0.011 mM. At 0.011 mM of PMAL-B-100, there was no S-CF expression of the tested GPCRs.

CF expression of GPCRs on a preparative scale often requires N-terminally fused Thioredoxin[21,26] or a 13-amino-acid-short T7-tag.[19,20,25,28] In this study, using a pIVEX vector, we have demonstrated the expression of CCR1, CCR5, CRFR1, and CRFR2β in preparative scale up to 1 mg/mL without any N-terminal modification (Table I). It has been speculated that the coding region of the N-terminal T7 tag allows efficient initiation of translation.[19] While pET vectors also contain a lac operator, the pIVEX system is based only on the bacteriophage T7 promoter and the ε translation enhancer. This constitutive promoter system in pIVEX vectors might reduce mRNA secondary structure formation within the nontranslated region, improving the initiation of translation and thus enhancing the CF expression of GPCRs without the need of N-terminal tags.

GPCRs were P-CF expressed and solubilized with LMPG; LMPG was subsequently exchanged to NVoy (P$_N$-CF)

In the absence of detergents, lipids, or polymer, CF-produced GPCRs precipitated in the RM and were harvested by centrifugation. We found that precipitated GPCRs could not be solubilized by up to 5 mM of NVoy alone. However, we observed that NVoy efficiently exchanged detergents of protein detergent complexes (PDCs) using affinity chromatography, for which insoluble GPCR precipitates were first solubilized by 1-myristoyl-2-hydroxy-sn-glycero-3-[phosphor-rac-(1-glycerol)] (LMPG). This procedure of GPCR precipitate solubilization in LMPG with subsequent exchange of LMPG to NVoy (P$_N$-CF) and purification of the soluble GPCR in NVoy resulted in more than 75% pure GPCR samples, as shown on SDS-PAGE in Figure 1(B), where proteins run as a mixture of monomers and dimers. Approximately 50–60% of the total CF-produced GPCRs can be purified and recovered by IMAC.

CRFR-NVoy complexes are homogenous and stable

Stability and homogeneity are indispensable prerequisites for functional and structural analysis of IMPs. Size exclusion chromatography (SEC) can determine protein quality by separating aggregated protein from nonaggregated protein fractions. CF-produced GPCRs in the presence of detergents, whether expressed in S$_D$-CF or in P-CF, tend to

Table I. *CF Expressed GPCRs Including a C-Terminal His$_6$ Tag*

Family	Origin	GPCR	Abbreviation[a]	N-term. tag[b]	MW (kDa)[c]	CF expression level (mg/mL)[d]
A	Human	C—C chemokine receptor type 1	CCR1	—	42.35	1
A	Human	C—C chemokine receptor type 5	CCR5	—	42.75	1
A	Human	Somatostatin receptor type 2	SSR2	T7	42.59	0.5
A	Human	Somatostatin receptor type 5	SSR5	T7	41.65	0.5
B	Human	Corticotropin-releasing factor receptor 1	CRFR1	—	46.88	1
B	Mouse	Corticotropin-releasing factor receptor type 2β	CRFR2β	—	48.87	1
B	Mouse	Corticotropin-releasing factor receptor type 2β	CRFR2β$_{T7}$	T7	50.21	0.2
C	Human	GPCR family C group 5 member B	GPRC5b	T7	44.61	0.7
C	Human	Retinoic acid-induced protein 3	RAI3	T7	43.22	0.1

[a] Used GPCR abbreviation.
[b] N-terminal fused tag.
[c] MW of GPCR including tags.
[d] CF expression level in P-CF, S$_D$-CF, and S$_N$-CF, determined by Western blot analysis.

aggregate, with over 75% of GPCRs ending up in the void volume during SEC.[27] In contrast, CRFR1 and CRFR2β obtained from P-CF, solubilized in LMPG that is subsequently exchanged to NVoy during IMAC purification, show almost no protein aggregation [Fig. 2(A,B)] and give the first evidence of the high level of homogeneity of the sample. Using SEC combined with ultra violet (UV), static light scattering (LS), and refractive index (RI) measurement (SEC-UV/LS/RI) with CRFR1 and CRFR2β, we were able to obtain protein and polymer molar masses in the protein-NVoy complex [Fig. 2(C)]. CRFR1 and CRFR2β form monomers with the estimated molar mass of 40.1 and 48.2 kDa, respectively. Approximately 20 NVoy molecules are bound to each CRFR molecule, accounting for the molecular weight of the polymer fraction of about 100 kDa.

Additionally, we used single particle analysis by electron microscopy (EM) to evaluate sample homogeneity. The analysis of both CRFRs in the presence of NVoy showed homogenous, monodispersed, and compact structures [indicated by arrows in Fig. 2(D,E)]. Single particle projections were extracted from the micrographs, reference aligned, and classified by multistatistical analysis to calculate class averages. Side view class averages of CRFR1 and CRFR2β display a pair of rods with the length of 6–7 nm and the center-to-center distance of 3–4 nm between the rods [Fig. 2(D,E)].

Stability and structural homogeneity of the protein sample were also estimated using NMR, which is a powerful tool to analyze structural quality of proteins at the molecular level. The spectral dispersion of the NMR signals gives information about the protein fold. Although the size of the protein drastically affects the signal line width in solution NMR, the [^{1}H,^{15}N]-TROSY-HSQC spectra of the ∼150 kDa U-^{15}N-CRFR2β-complex with NVoy, produced by S$_N$-CF and measured at pH 4.0 and 310 K, show a set of about 100 sharp peaks well dispersed between 7.7 and 8.7 ppm in the proton dimension [Fig. 2(F)].

Polymer-solubilized CRFRs have ligand-binding specificity

Demonstrating protein function provides strong evidence of the quality of protein fold. We analyzed the ability of purified CRFR1 and CRFR2β to bind different agonists and antagonists. CRFR1 and CRFR2β were produced by S$_N$-CF or P$_N$-CF, purified and tested in NVoy. Additionally, CRFR2β produced by P-CF was solubilized, purified, and tested in LMPG. We tested the CRFR antagonist astressin and the agonists rat urocortin 1 (rUcn1), mouse urocortin 2 (mUcn2), and mouse urocortin 3 (mUcn3). The detailed binding data for the four tested ligands are given in Table II. CRFR2β purified from S$_N$-CF showed nanomolar binding to rUcn1, astressin, and mUcn2 [Fig. 3(A), Table II], whereas mUcn3 bind with lower affinity (Table II). CRFR2β prepared by S$_N$-CF and that prepared by P$_N$-CF both showed similar binding to astressin, estimated at 10 n*M* [Fig. 3(B)]. CRFR1 produced by P$_N$-CF also showed affinity for astressin similar to that of CRFR2β [Fig. 3(B)]. In addition, the CRFR agonist sauvagine showed very low affinity (>500 n*M*) with little competitive displacement of labeled astressin for all samples [Fig. 3(C)].

The fraction of active receptors was estimated based on the total specific binding and assuming that the K_d for astressin bound to the receptor expressed in NVoy was the same as the K_d for astressin bound to receptors expressed in mammalian cells, and a 1:1 ligand:receptor ratio. The purified samples contained at most 10% of CRFR2β capable of binding ligands. There was no correlation between the amount of functional receptor and the
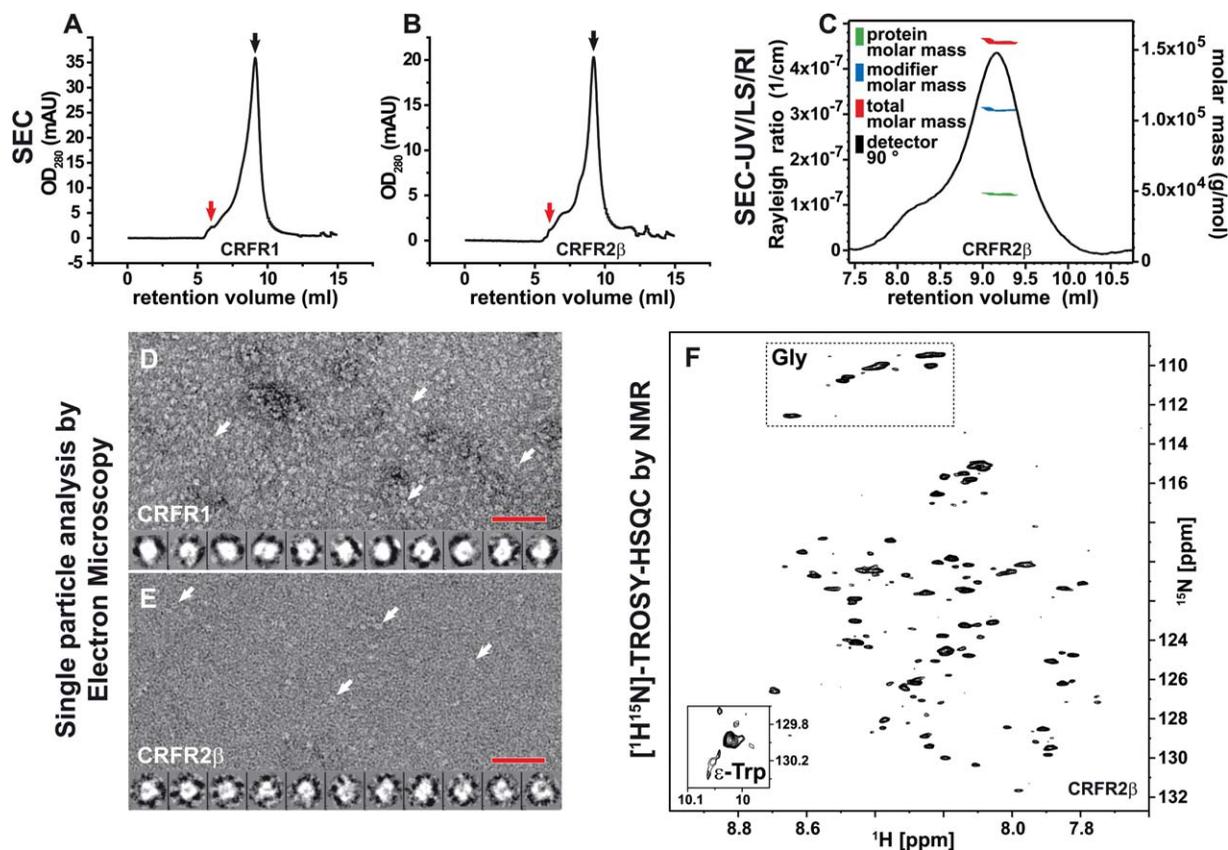
Figure 2. Protein analysis of CF-expressed CRFR1 and CRFR2β in the presence of NVoy. CRFR1 (A) and CRFR2β (B) obtained from P_N-CF were analyzed by SEC. The aggregated fraction (red) and monomeric fraction (black) are indicated by arrows. C: 90° LS detector signal from SEC-UV/LS/RI analysis of CRFR2β in NVoy with a given molar mass distribution between CRFR2β (green), polymer (blue), and their complex (red). CRFR1 (D) and CRFR2β (E) expressed by P_N-CF were analyzed by single particle analysis. Electron micrographs of negatively stained overviews show monodispers nonaggregated particles (indicated by arrows). The corresponding side view class averages are at the bottom. The scale bars (red) correspond to 60 nm in the overviews and the size of each average is 16 nm. F: [^{1}H,^{15}N]-TROSY-HSQC spectra of CRFR2β in NVoy. Approximately 20 μM U-^{15}N-CRFR2β, produced by S_N-CF, in ∼1 mM NVoy, 10 mM NaCl, 20 mM MES/Bis-Tris (pH 4.0) were measured at 310 K on a 700 MHz spectrometer equipped with a cryogenic probe. Characteristic Trp N$^\varepsilon$^{1}H and Gly H^N cross peaks are indicated.

expression mode. The different receptor samples in NVoy were functionally stable for weeks at 4°C and showed no decrease in binding affinity after storage. The active fraction of CRFR2b is easily purified with the astressin-Affi-Gel 15 resin. The receptor is bound to the resin, the nonspecifically bound fraction is washed off with high salt buffer, and all of the specifically bound CRFR2b is eluted with low pH. Relative intensities of the Western blot bands showing fractions of the purified proteins [Fig. 3(D)] demonstrate that ∼10% of the total sample is bound to the astressin resin, confirming the similar estimation of the active fraction from the ligand-binding study. Given the CRFR CF expression level of 1 mg

Table II. *Inhibitory Binding Constants, K_i (nM) for Various CRF Family Ligands Binding to CF Produced CRF Receptors Measured by Competitive Displacement of ^{125}I-[D-Tyr^0]astressin*

Receptor	Preparation	Astressin	rUcn1	mUcn2	mUcn3
CRFR2β	P_L-CF[a]	8.7 (4.5–16.9)	4.0 (3.6–4.4)	51 (50–52)	461 (117–1800)
CRFR2β	S_N-CF[b]	11.9 (10.6–13.3)	4.8 (3.4–6.7)	35.2 (24.2–51.4)	317 (55–1830)
CRFR2β	P_N-CF[c]	9.9 (7.2–13.7)	5.9 (5.2–6.8)	74 (64–84)	205 (176–238)
CRFR1	S_N-CF	27 (12–64)	46 (27–79)	>1000	>1000
CRFR1	P_N-CF	8.3 (5.1–13.4)	13.9 (11.3–17)	492 (433–560)	>1000

The average of at least three independent experiments is given together with 95% confidence limits shown in parentheses.
[a] P_L-CF, solubilized in LMPG and purified.
[b] S_N-CF, soluble CF expressed and purified in surfactant NVoy.
[c] P_N-CF, solubilized in LMPG, exchanged to NVoy and purified.

Polymer-Based Cell-Free Expression of GPCRs
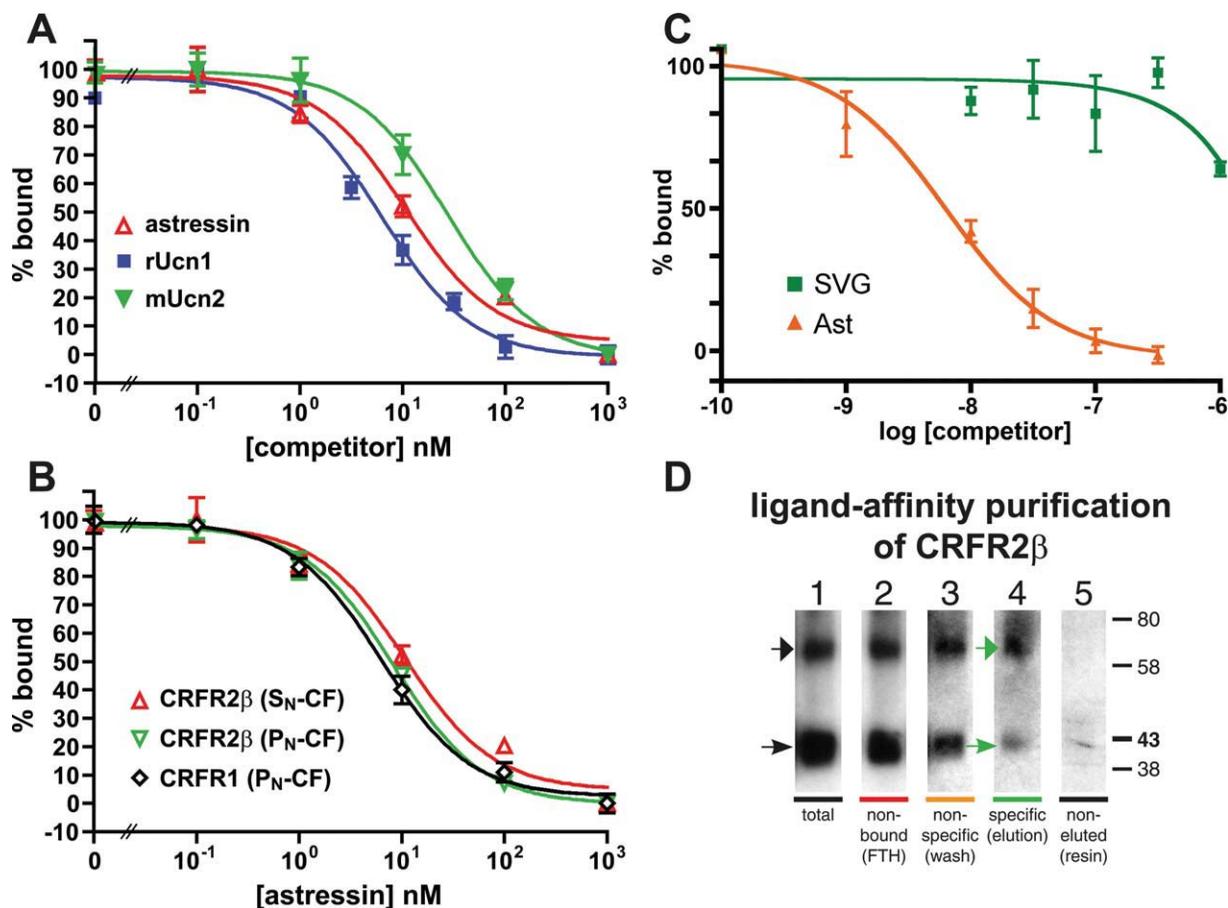
Figure 3. Ligand binding of CF expressed CRFR1 and CRFR2β in the presence of NVoy. A: Competitive displacements of [^{125}I-D-Tyr^0]astressin, bound to CRFR2β from S_N-CF, with the ligands astressin (red), rUcn1 (blue) and mUcn2 (green). B: Competitive displacement of ^{125}I-[D-Tyr^0]astressin by astressin is compared for different CF preparations. CRFR2β was expressed by S_N-CF (red) and P_N-CF (green). In addition CRFR1 from P_N-CF (black) is shown. Error bars of three measurements are indicated. C: Comparison of competitive displacement of ^{125}I-[D-Tyr^0]astressin by astressin and sauvagine. D: Ligand-affinity purification of functional fraction from P_N-CF produced CRFR2β. Total amount of receptor loaded on astressin-Affi-Gel 15 (1), nonbound receptor fraction in flow through (FTH) (2), nonspecifically bound receptor in wash fraction (wash) (3), specifically bound receptor, eluted by low pH (4), and noneluted receptor on resin (5) are verified by Western blot against the C-terminal His$_6$-tag of CRFR2β.

per mL of the CF RM and its 50–60% recovery from subsequent purification, of which ~10% constitutes the active fraction, ~50 µg pure, active CRFR can be obtained from 1 mL of the CF RM. This means that 1 mg of pure, functional receptor can time- and cost-effectively produced within 2 days at a cost less than $1500 by our optimized CF expression system.[43]

Discussion

Detergent can be substituted by polymer in membrane protein studies

Detergents are often required in the biochemical analysis of IMPs. However, detergent-solubilized proteins tend to aggregate.[30] One way to bypass the use of detergents is employing APs.[32] NVoy shares some properties of detergents and APs, but in contrast to common APs like A8-35[32] and PMAL-B-100,[34] which are both polyacrylate-derived, of limited

solubility, and charged, NVoy is neutral and highly soluble in aqueous solution due to its polyfructose backbone. NVoy is also more soluble and forms larger micelle-like structures compared with the recently reported glucose-based amphiphilic telomers.[38,39]

So far, the membrane extraction of IMPs has been limited to the use of detergents. To our knowledge, APs have not been shown to extract IMPs from native membranes. We also failed to solubilize IMPs, such as Etk and YcjF-GFP, in PMAL-B-100. However, by using NVoy at concentrations above 3 mM, we have demonstrated the successful extraction of Etk and YcjF-GFP from *E. coli* membranes. Whereas YcjF-GFP can be extracted by many detergents, Etk can only be extracted by phosphocholine-derived detergents. Thus, NVoy and possibly other NVoy-like polymers might have universal applicability for IMP extraction.

Globular proteins are often influenced by detergent environment. For instance, the catalytic

activity of tobacco etch virus protease is often inhibited in the presence of detergents.[40] In contrast, we have shown the successful cleavage of YcjF-GFP in the presence of NVoy at 5 mM.

GPCRs can be expressed in a detergent-free, soluble CF expression system

CF expression has emerged as an alternative to conventional cell-based heterologous expression systems, which is capable of producing functional and preparative amounts of large eukaryotic transporters[41] and several family A GPCR members.[21,26–28] In addition, CF expression has been successfully used to crystallize human VDAC[42] and to determine by NMR membrane domain structures of three classes of histidine kinase receptors[43] and the C-terminal fragment of presenilin 1.[44] A complete sample preparation by CF expression takes less than 24 h including overnight sample incubation. The open nature of CF systems also allows flexibility in the choice of additives to RM, which in turn has permitted different modes of CF synthesis of IMP.

NVoy, which is a nondetergent polymer and, as we have shown, does not interfere with the transcriptional and translational machinery, provides a new S-CF mode for IMPs (S_N-CF), in addition to S_D-CF[19,21] and S_L-CF.[22–24] As demonstrated in the Results section, we have successfully used the S_N-CF mode for all three major GPCR families.

CRFR samples are homogenous and not aggregated in the presence of NVoy

Detergents often destabilize solubilized IMPs and promote nonspecific protein aggregation.[30] For CF expression in particular, it has been reported that without addition of strong reducing agents, olfactory receptors were found to be highly aggregated in all tested detergent and buffer conditions.[27] However, using SEC analysis, we were able to demonstrate a high level of sample homogeneity and almost no aggregation of the P_N-CF-expressed CRFRs. In addition, single particle analysis of the P_N-CF-produced CRFR1 and CRFR2β in the presence of NVoy reveals homogenous distribution of particles. The observed surface topologies on carbon-coated copper grids in EM predominantly indicate the formation of homodimers with a minor amount of monomeric receptors and show no aggregation. We assume that this dimer formation is promoted by the absorption process of diluted receptor samples on grids, used for EM analysis. Furthermore, NVoy can aid the single particle analysis by Cryo-EM, whereas the analysis of detergent-solubilized membrane proteins is often hindered by the fact that detergents reduce the surface tension of water, hampering the control of ice thickness, and the distribution of protein.[45]

The solution NMR analysis of GPCRs has so far been limited by the inefficiency of cellular expression systems in the preparation of isotopically labeled samples and by massive line broadening resulting from large PDC sizes and the intrinsic internal mobility of the TM part, as demonstrated in Supporting Information Figure S2 for CRFR2β in LMPG micelles. In contrast to the detergent solubilized sample, U-^{15}N-labeled CRFR2β produced by S_N-CF shows sharp cross peaks in [^{1}H,^{15}N]-TROSY-HSQC spectra for ~25% of the CRFR2β amino acids. From a comparison of the number of cross peaks in the glycine and the tryptophan side chain regions of the spectra with the number of glycine and tryptophan residues in the C-terminal and the extracellular domains of the receptor, as well as from the comparison of the obtained spectra with the published spectra of the ECD1,[46] we conclude that the residues represented by the cross peaks in our spectra most likely belong to the flexible part of the ECD1 and the C-terminus. Other signals are completely broadened because the rest of the molecule is buried within the massive ~100 kDa NVoy micelle. Nevertheless, the observed spectral dispersion of the signals in [^{1}H,^{15}N]-TROSY-HSQC spectra gives evidence of folded and stable "NMR-visible" CRFR2β regions.

CRFR samples in the presence of NVoy show selective ligand binding activity

In contrast to family A GPCRs, where ligands bind to the TM domain, CRFRs are targeted by diverse ligands that bind to the extracellular domains. Family A GPCRs have been previously expressed in CF systems and functionally analyzed.[21,25–28] In this study, we have analyzed the ability of CF expressed CRFR1 and CRFR2β to bind different ligands and were able to show specific ligand binding of these two family B GPCRs. This is especially important as CRFRs possess a relatively large ECD1, which is involved in ligand binding, and it is a common problem that a detergent distorts the folding of the hydrophilic domains of detergent-solubilized proteins. The CRFR antagonist astressin and agonist rUcn1 have been shown to bind both CRFRs,[47] whereas the agonists mUcn2 and mUcn3 selectively bind type 2 CRFRs.[48] CF-expressed CRFR1 and CRFR2β show nanomolar binding to astressin and rUcn1 in the presence of NVoy. Furthermore, we measured selective binding of mUcn2 to CRFR2β, demonstrating the high quality of the CF produced CRFR samples. The inhibitory binding constants we measured are comparable with those obtained for individually expressed ECD1 and 1–2 orders of magnitude higher than the constants measured for receptors in their native environment of CHO membrane fractions.[49,50] These results suggest a correct folding of the ECD1. As we have previously demonstrated the proper folding of the TM region of CF-expressed family A GPCRs, we can presume that the

TM domains of CF-expressed family B GPCRs are also properly folded. This is further supported by the fact that ECD1 of the family B GPCRs are functional and there are spatial relations between the ECD and TM regions in the protein. However, as none of the tested ligands targets the TM region, the proper folding of the TM segments cannot be firmly concluded based on the obtained data and still needs to be experimentally addressed. The observed affinities of the agonists mUcn2, mUcn3, and sauvagine for CF produced CRFR samples are significantly lower than that of astressin and rUcn1. However, it has been shown that the affinity of some agonists to CRFRs is increased in the presence of G-proteins.[51] In particular, the CRFR2 binding to Ucn2 and Ucn3 was decreased in the absence of G-proteins, whereas binding to Ucn1 was not affected.[52] We also observed high affinity CRFR2β binding to rUcn1 but reduced affinity to mUcn2 and mUcn3. Thus, the lower affinity for some of the agonists may result from the absence of G-proteins. For sauvagine and some CRF ligands, ECD1 glycosylation of CRFR1 has been reported to be important for binding of some ligands.[53] This could explain the reduced sauvagine affinity to CF expressed nonglycosylated receptors, although reduced binding of some agonists could also be a result of the presence of detergent or polymer as it is widely accepted that detergents may affect the folding of IMPs and distort TM regions. Also, the detergent may bind to the ligands and may shield them from interacting with the receptor.

Purification of soluble CF-expressed GPCRs in the presence of detergents is often impeded by the presence of *E. coli* extract proteins.[28] The CF expression of GPCRs as precipitate and their subsequent solubilization in LMPG are the first purification step. As NVoy completely exchanges LMPG on IMAC (P_N-CF), which constitutes the next step of protein purification, we can obtain pure GPCRs in a desirable polymer environment. When compared with S_N-CF expression, the samples from P_N-CF show similar (CRFR2β) or better (CRFR1) ligand binding (Table II) with the benefit of enhanced purity.

Conclusion

This study shows the first CF expression of members of all three GPCR families and demonstrates ligand binding for B family GPCRs. The use of NVoy allowed us to overcome detergent related aggregation problems and enabled the production of nonaggregated high quality samples from P_N-CF and the newly introduced S_N-CF mode. We showed that NVoy extracted IMPs from the membrane without influencing globular proteins, demonstrating general applicability of this polymer for IMPs. CF systems are still a new avenue for expressing IMPs, but we showed its applicability to difficult IMPs, such as GPCRs, which can now be synthesized in prepara-

tive amounts. A common problem with detergent-solubilized membrane proteins is that the balance between detergent concentration which needs to be high enough to keep the protein soluble and yet low enough not to affect the folding of its hydrophilic domains, is very difficult to achieve. With these results, we show that nondetergent-like polymers can easily keep that balance and may be an excellent alternative to surfactants. They may improve protein stability and maintain the protein's function, thereby enabling new possibilities for functional and structural studies of GPCRs and difficult to obtain IMPs in general.

Materials and Methods

CF expression

GPCRs were produced in an individual CECF system according to previously described protocols[16,17,19] with further optimizations. Chemicals for CF expression were purchased from Sigma-Aldrich unless otherwise stated. CF extracts were prepared from *E. coli* strain A19 after previously described protocols[16,17] and T7-RNA polymerase was expressed using the pT7-911Q plasmid[54] and purified as described elsewhere.[55] Preparative scale CF reactions were performed in 25 kDa MWCO dispodialyzers (Spectrum Laboratories, CA) using 1 mL or 5 mL of RM set with a volume ratio between RM:feeding mixture (FM) of 1:17. Dispodialyzers were placed in suitable plastic tubes holding the FM and incubated for ~18 h on a roller device (Labortechnik Froebel GmbH, Germany). The reaction conditions for the CF reaction were as follows. RM and FM: 270 mM potassium acetate; 14.5 mM magnesium acetate; 100 mM Hepes-KOH pH 8.0; 3.5 mM Tris-acetate pH 8.2; 0.2 mM folinic acid; 0.05% sodium azide; 2% polyethyleneglycol 8000; 2 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCP) (Pierce, IL); 1.2 mM ATP; 0.8 mM each of CTP, UTP, GTP; 20 mM acetyl phosphate (Fluka, Germany); 20 mM phosphoenol pyruvate (AppliChem GmbH, Germany); 1 tablet per 50 mL complete protease inhibitor (Roche Applied Science, IN); an GPCR amino acid distribution optimized amino acid mixture containing 2.5 mM of the amino acids I, L, F, V; 1.875 mM A, R, N, Q, G, K, S, T, Y; 1.25 mM D, C, E, H, M, P, W in the FM; RM: 1.5 mM of the amino acids I, L, F, V; 1.125 mM A, R, N, Q, G, K, S, T, Y; 0.75 mM D, C, E, H, M, P, W; 40 μg/mL pyruvate kinase (Roche Applied Science, IN); 500 μg/mL *E. coli* tRNA (Roche Applied Science, IN), 0.3 U/μL RNase Inhibitor (SUPERase-In™, Ambion, TX); 0.5 U/μL T7-RNA polymerase; 40% S30 extract and 15 μg/mL of pET21a derived plasmid DNA or 7.5 μg/mL of pIVEX2.3 derived plasmid DNA. S-CF was performed in the presence of 0.5 mM NVoy (Expedeon Protein Solutions, UK) in RM and 0.05 mM NVoy in

FM. For testing, the AP polymer PMAL-B-100 (Cal-biochem, NJ), 0.011 mM was used in RM and FM.

For CF expression as precipitate (P-CF), a precipitate containing recombinant proteins expressed in the absence of detergents was removed from the RM by centrifugation at 20,000g for 10 min and suspended in 50% volume equal to the RM volume in 2% (w/v) LMPG, 20 mM Tris-HCl pH 7.4, 500 mM NaCl. The suspension was incubated for 30 min shaking at 1000 rpm at 25°C followed by centrifugation at 20,000g for 10 min to remove residual precipitate. The supernatant was diluted 1:10 in dilution buffer ready for affinity purification.

For CF U-^{15}N labeling, RM and FM were supplemented with 0.5 mM of ^{15}N algal amino acid mixture (CIL, MA) and 1 mM of the ^{15}N amino acids N, C, Q, W (CIL, MA).

GPCR purification and reconstitution

Precipitate was removed from the RM after CF expression by centrifugation at 20,000g for 10 min. After expression in soluble mode in the presence of NVoy, the RM was centrifuged at 20,000g for 10 min and the supernatant was diluted 1:10 in dilution buffer (100 mM Tris-HCl pH 7.4, 500 mM NaCl, 20 mM imidazole) to reduce NVoy concentration from 0.5 mM to 0.05 mM. Diluted solubilized precipitates and RMs were applied to 1 mL Co^{2+}-loaded HiTrap HP columns (GE Healthcare, NJ) equilibrated in column buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl) supplemented with 20 mM imidazol and the corresponding additive, 0.01% 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG) (Avanti Lipids, AL) or 0.05 mM NVoy. Affinity chromatography was performed with 1 mL/min flow rate with washing steps of six column volumes of column buffer supplemented with 20 and 50 mM imidazol, respectively. Bound protein was finally eluted with 375 mM imidazol in column buffer and 0.3 mL fractions were collected. In case of LMPG to NVoy exchange, CoITA columns were washed with 20 column volumes of column buffer supplemented with 20 and 50 mM imidazol, respectively. For buffer exchange, protein containing fractions were dialyzed in 3.5 kDa MWCO Slide-A-Lyzer Dialysis Cassettes (Pierce, IL) using the corresponding buffer and detergent, NVoy was not added to dialysis buffer.

SEC-UV/LS/RI analysis

The analyses of NVoy (NVoy = NV10, Expedeon Protein Solutions, UK) and NVoy—protein complexes were performed by measuring the relative RI signal (Optilab rEX, Wyatt Technology Corporation, CA), LS signals from three angles (45°, 90°, 135°) (mini-DAWN, Wyatt Technology Corporation, CA), UV extinction at 280 nm (Waters[TM] 996 Photoiode Array Detector, Millipore Corporation, MA) combined with an analytical polymer-based size exclusion column (Shodex® Protein KW-803, Waters Corporation, MA) on an HPLC instrument (Waters[TM] 626 Pump, 600S Controller, Millipore Corporation, MA). The data were analyzed using the Astra V 5.3.2.12 Software (Wyatt Technology Corporation, CA). The mathematical background for LS, RI, and UV for determine the IMP weight by this setup is extensively described elsewhere.[56]

dn/dc_{NVoy} was determined as described elsewhere[57] by analyzing the RI of different polymer concentrations using the Astra template file for dn/dc determination after injecting 3 mL of 0, 0.05, 0.1, 0.25, 0.3, 0.4, 0.5, and 0.6 mM NVoy solubilized in running buffer (20 mM Tris-HCl pH 7.4, 300 mM NaCl) into running buffer at 1 mL/min flow rate with 10 mL offset between injections. All solutions were filtered with 0.2 μm Nylon membrane filters (PALL Life Sciences, MI). The polymer complex size was measured by injecting 0.2 mM NVoy into water at 0.8 mL/min flow rate at the given setup. GPCRs were analyzed by injecting purified protein into HPLC buffer (20 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.02 mM NVoy) at 0.8 mL/min. After baseline adjustment, alignment of the measurements and band broadening correction, the data was analyzed by applying the Astra protein conjugate template.

Single particle analysis

Different concentrations of GPCR particles were adsorbed onto 400 mesh carbon-coated copper grids (Ted Pella, CA), negatively stained with 2% (w/v) uranyl acetate, blotted, and air-dried. A constant NVoy concentration was kept during the dilutions. Grids were imaged in a JEOL JEM-2100F transmission electron microscope (JEOL, Japan) equipped with a field-emission gun under low-dose conditions. Images were recorded at a nominal magnification of 50,000× under minimum dose procedures on a Tietz 4096 × 4096 pixel CCD camera (TVIPS, Germany), resulting in a pixel size of 2 Å. Between 200 and 2000 particles were selected for each GPCR using the EMAN boxer program.[58] Reference-free alignment of the particles followed by Singular Value Decomposition and k-means classification were performed iteratively with the EMAN package[58] to obtain the final class averages.

NMR measurement

NMR samples of CRFR2β from S$_N$-CF were purified by Co^{2+}-loaded HiTrap HP columns on FPLC as described above and dialyzed against 20 mM MES-BisTris pH 4.0, 10 mM NaCl overnight. The samples were concentrated to 300 μL in 30 kDa MWCO Vivaspin 2 concentrators (Sartorius Stedim Biotech GmbH, Germany) and supplemented with 5% D$_2$O and 0.5 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). [^{15}N, ^{1}H] TROSY spectra were obtained on a Bruker 700 MHz spectrometer equipped with

four radiofrequency channels and a triple-resonance Cryo-probe with a shielded z-gradient coil. CRFR2β in NVoy was measured at 310 K with 256 scans.

Radioreceptor assays and affinity purification

Soluble proteins were incubated in triplicate wells with $[^{125}I\text{-}DTyr^0]$-astressin ($\sim$150,000 cpm), together with increasing concentrations of unlabeled peptide (astressin, rUcn1, mUcn2, mUcn3, sauvagine), in 0.2 mL assay buffer (50 mM Hepes pH 7.5, 0.1% (w/v) BSA, 0.005% TritonX-100) for 90 min at RT in MultiScreen$_{HTS}$ GV microtiter plates (Millipore, Germany) precoated with 0.1% (w/v) polyethylenimine. In the case of assays using receptors in LMPG, the buffer was adjusted to 0.01% detergent to maintain the critical micellar concentration. The mixture was aspirated under vacuum, and the plates were washed twice with 50 μL of assay buffer. Counts bound in the well were quantified by γ-counting. The K_i's were calculated by PRISM (GraphPad, CA) fitting program. All assays were performed at least three times in triplicate. The fraction of active receptors was calculated assuming the K_d for the receptor was the same as the K_d for astressin bound to the receptor expressed in mammalian cells, assuming a 1:1 receptor:ligand complex and assuming a MW of 40 kDa for the receptor. For affinity purification, 80 μg CRFR2β were bound to an astressin affinity gel prepared by coupling astressin to Affi-Gel 15 (Bio-Rad Labs, CA), using dimethylformamide according to company's procedure. A total of 100 μL CRFR2β from P$_N$-CF were diluted 10 times in dilution buffer (20 mM TRis-HCl pH 7.4, 50 mM NaCl, 0.05 mM NVoy) and incubated with 50 μL astressin-Affi-Gel 15 for 3 h at RT. Subsequently, the affinity gel was washed three times with 100 μL dilution buffer and protein was eluted at low pH (20 mM MES pH 4, 50 mM NaCl, 0.05 mM NVoy) in five steps of 100 μL each. The result was analyzed by Western blot.

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