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Improved yield of a ligand-binding GPCR expressed in *E. coli* for structural studies

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ABSTRACT

The G-protein coupled receptor (GPCR), rat brain neurotensin receptor type I (NTS1) is one of a small number of GPCRs that have been successfully expressed in *Escherichia coli* as a functional, ligand-binding receptor, but yields of purified receptor are still low for comprehensive structural studies. Here, several approaches have been examined to optimize the yields of active, ligand-binding receptor. Optimisation of *E. coli* strain and induction protocol yielded a significant improvement in expression of active receptor. Expression of the receptor in BL21(DE3) cells, in combination with autoinduction improved expression 10-fold when compared with previously reported expression protocols using IPTG-mediated induction in DH5α cells. Optimization of the purification protocol revealed that supplementation of buffers with phospholipids enhanced recovery of active receptor. The methods examined are potentially applicable to other GPCRs expressed in *E. coli*.

Introduction

G-protein coupled receptors (GPCRs)[1] are integral membrane proteins with seven transmembrane spanning helices [1]. They transduce extracellular stimuli across the cell membrane to active heterotrimeric G-proteins which, in turn, act on downstream effector pathways. Over 800 members of the G-protein coupled receptor (GPCR) superfamily have been identified in the human genome [2,3]. Their critical role in sensory, neuromodulatory and homeostatic pathways makes them a major pharmacological target, and over 30% of current drugs target GPCRs [4]. Until recently there was only one example of this family in the Protein Data Bank, that of bovine rhodopsin—a non-ligand-binding, retinal activated photoreceptor [5]. The crystal structures of the human β2 adrenergic receptor represent a major advance in this field [6,7]. However, low sequence homology within this family presents a barrier to effective structure-based drug design [8], consequently the structural elucidation of GPCRs remains one of the most pressing goals facing structural biology today [1].

For structural studies of membrane receptors, which are typically expressed at a low level in their native environment, expression as a recombinant protein in *Escherichia coli* is a powerful tool, with the potential for high yields of protein and straightforward incorporation of specific labelled amino acids or stable isotopes where required. For many membrane proteins, particularly those of eukaryotic origin, expression in such heterologous systems has not been achieved, presenting a major bottleneck in this field of structural biology [1].

The neurotensin receptor type I (NTS1) is one of a few examples of GPCRs that have been expressed as a functionally ligand-binding protein in *E. coli* [9–22]. NTS1 is expressed as a fusion protein N-terminally tagged with maltose binding protein (MBP) and C-terminally with thiorodoxin and His_{10} (termed NTS1A) under the control of the of the *lac* operator/promoter. Expressed in DH5α cells at 20 °C for 40 h, yields of 0.2–0.4 mg/L of active receptor can be obtained [23–25]. This exact construct has been successfully used to express other class A GPCRs: CB2 cannabinoid receptor [11], M_2 muscarinic acetylcholine receptor [13] and the human adenosine A(2a) receptor [9], as folded, ligand-binding membrane proteins.

In this work we focused on induction protocol and bacterial strain for the optimization of expression. NTS1A and NTS1B (an equivalent construct with Tev cleavage sites [25]) have been traditionally expressed in DH5α [10], a strain not typically used for protein expression. A variety of *E. coli* strains have been developed for expression, most are derived from the *lon* and *ompT* protease deficient strain BL21 [26]. Strains that harbour auxiliary plasmids, such as those encoding tRNAs to read rare codons (pRARE) or promote disulphide bond formation (such as the Origami series), have

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[1] *Abbreviations used:* CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHS, cholesterol hemisuccinate; DDM, dodecyl-β-D-maltoside; GPCR, GTP-binding protein-coupled receptor; IPTG, Isopropyl β-D-1-thiogalactopyranoside; MBP, *E. coli* maltose binding protein; NT, neurotensin; NTS1, neurotensin receptor type 1; NTS1A, MBP-rT43NTS1-Trx-H10; NTS1B, MBP-TeV-rT43NTS1-Trx-TeV-H10; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; TeV, tobacco etch virus; Trx, thiorodoxin.

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also been shown to increase the expression of eukaryotic proteins [27] and two *E. coli* cell lines, C41 and C43, selected from the parental cell line BL21, have been developed which are adapted to the expression of membrane and toxic proteins [28].

Over-expression of heterologous proteins in *E. coli* is usually tightly controlled using an inducible promoter [26]. The majority of *E. coli* expression systems employ the *lac* promoter coupled with IPTG-mediated induction. In these systems, even when combined with repression mediated via the *lac* operator sequence [29], some background expression may occur. Leaky expression of toxic genes, such as membrane proteins may, in turn, lead to lower levels of protein expression upon induction due to plasmid instability or cell stress [30]. Autoinduction, an alternative to IPTG-induced expression that relies on catabolite (glucose) repression and substrate (lactose) induction to provide the off-on switch for protein expression, can provide extremely tight control of expression. Coupled with the reduced need for sample handling (no OD_{600}-dependent induction window) and ease with which the culture size can be scaled-up, this system is attractive for high-throughput expression screening [31]. Furthermore, autoinduction allows cells to grow to high density prior to induction often resulting in higher yields of protein [32]. As over-expression of membrane proteins into the bacterial membrane is often associated with host cell toxicity, autoinduction offers obvious benefits and an expression trial of membrane proteins from *Mycobacterium tuberculosis* in *E. coli* found that in 75% of cases expression was increased when compared with IPTG-mediated induction [33].

Detergents can remove integral lipids from membrane proteins during the solubilisation and purification process, with concomitant potential for reduction in receptor activity, presumably due to protein unfolding or partial denaturation [34,35]. Thus, supplementing the purification media with phospholipids may help to stabilize proteins and retain their active conformation. Here, the way in which different combinations of detergents and phospholipids affect the activity during the solubilisation and purification of NTS1 was investigated.

Many structural genomics projects have relied on eukaryotic expression systems to produce authentically folded GPCRs and in *E. coli* use strong promoters to express GPCRs in inclusion bodies. Although this strategy can result in large amounts [36] of protein, refolding GPCRs is a major challenge and only three GPCRs have been successfully refolded from inclusion bodies [37–39]. To date, 15 GPCRs have been expressed in *E. coli* as functional, ligand-binding receptors inserted into the membrane [9–21]. However, many are expressed at low levels (less than 0.1 mg/L culture [36]); and few, such as the cannabinoid CB2 receptor [12] (1 mg/L), have achieved levels amenable to comprehensive structural studies [36]. The data presented here shows that there is significant scope and potential for improvement in the yield of functionally competent receptor. Such methods described here may be useful for expression screening and production of other GPCRs in *E. coli*.

Materials and methods

Expression of NTS1B

The NTS1B fusion construct has been described previously by Grisshammer et al. [23,40]. This constructs encodes the rat NTS1 receptor with residues 1–43 truncated, an N-terminal fusion of the maltose binding protein (MBP) (including periplasmic targeting sequence) and a C-terminal thiorodoxin and deca-histidine tag (MBP-NTS1B-Trx-His_{10}). TeV protease sites enable the proteolytic removal of the MBP and Trx-His_{10} tags, which can subsequently be separated from the receptor by gel filtration. The presence of the TeV cleavage sites does not influence expression levels [25].

The NTS1B plasmid was transformed into either DH5α, BL21(DE3), Rosetta2 (BL21(DE3) transformed with pRARE2) (Novagen), C41(DE3), or C43(DE3) (Cambridge Bioscience, Lucigen). Cells were plated onto LB-agar plus 100 μg/ml ampicillin (and 34 μg/ml chloramphenicol for Rosetta2) and 1% (*w/v*) glucose to suppress expression. For the induction of protein expression with IPTG, 0.5 ml of an overnight culture (LB supplemented with 1% (*w/v*) glucose and antibiotics) was used to innoculate 50 ml of 2xYT media supplemented with 0.2% (*w/v*) glucose in a 250 ml flask and incubated at 37 °C with shaking at 200 rpm (Innova 4430 shaking incubator, New Brunswick Scientific). When an OD_{600} of 0.35 was reached the cultures were cooled to 20 °C and, when the OD_{600} = 0.5, induced with IPTG. Cells were harvested by centrifugation 30 h after induction (protein expression was not found to increase beyond this time point, data not shown), flash frozen in liquid N_2 and stored at −80 °C.

For autoinduction three media formulations were tested: MagicMedia™ (Invitrogen; for details see Nature Application notes [41], http://www.nature.com/app_notes/nmeth/2007/071707/pdf/an2808.pdf), Overnight Express™ Autoinduction System 2 (Novagen; for details see Novagen I*nov*ations Newsletter 21 [42], http://www.merckbiosciences.co.uk/product/240021) and a homemade autoinduction media, ZYP-5052 rich media, based on the method developed by Studier [32]. For 50 ml of ZYP-5052 rich autoinduction media 50 μl 1 mM $MgSO_4$, 50 μl 1000× trace metals (50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 2 mM $CoCl_2$, 2 mM $CuCl_2$, 2 mM $NiCl_2$, 2 mM Na_2MoO_4, 2 mM Na_2SeO_3, 2 mM H_3BO_3), 1 ml 50 × 5052 (25% (*w/v*) glycerol, 2.5% (*w/v*) glucose, 1% (*w/v*) lactose, freshly prepared) and 2.5 ml 20× NPS (0.5 M $(NH_4)_2SO_4$, 1 M KH_2PO4, 1 M Na_2HPO_4) were added to 46.5 ml ZY (10 g tryptone (Oxoid), 5 g yeast extract (Melford) per L). Fifty microlitres of an overnight culture was used to innoculate 50 ml autoinduction media in a 250 ml flask. Cultures were grown at 26 °C for 24 h with shaking at 200 rpm.

Small scale solubilisation of NTS1B

NTS1B was purified using a protocol based on that reported by Grisshammer et al. [25]. All operations were carried out at 4 °C unless otherwise stated.

For expression trials, 10 ml of cell culture was harvested by centrifugation, snap frozen in liquid N_2 and stored at −80 °C. The cell pellet was resuspended in 2× solubilisation buffer (100 mM Tris pH 7.4, 400 mM NaCl, 60% glycerol (*v/v*)). Two-hundred microlitres of 2× solubilisation buffer was used for cultures with a final OD_{600} below 2 and 400 μl for those greater than 2. About 120 or 240 μl of dH_2O, respectively, was added. DNase I (1 U/ml), lysozyme (1 mg/ml) and the protease inhibitors leupeptin (1 μg/ml), aprotinin (1 μg/ml) and pepstatin A (1 μg/ml) were added and the sample incubated on ice for 30 min. To lyse the cells and solubilize membrane proteins, detergents DDM, CHAPS and CHS were added to a final concentration of 1%, 0.5% and 0.1% (*w/v*), respectively. The sample was placed on a rotating wheel at 4 °C for 1 h. Cell debris was pelleted by centrifugation at 30,000*g*. The solubilized sample was snap frozen and stored at −80 °C.

Phospholipid supplementation

For a small-scale trial, NTS1B was expressed in BL21(DE3) for 24 h at 26 °C in 250 ml MagicMedia™ in a 2.5 L baffled flask (Nalgene polycarbonate baffled flasks were found to produce the highest expression), with shaking at 200 rpm. For receptor solubilization, 1 g of cell pellet was resuspended in 2 ml solubilisation buffer. DNase I, lysozyme and the protease inhibitors were added (as above) and the sample incubated on ice for 30 min with stirring. DDM, CHAPS and CHS were added to a final concentration

of 1%, 0.5% and 0.1% (*w*/*v*), respectively. For extraction with only DDM/CHS, CHAPS was excluded. The sample was made up to 4 ml with dH_2O and stirred for 1 h, followed by sonication for 30 s. Cell debris was pelleted at 60,000*g* for 1 h. Detergent solubilized fraction was filtered through a 0.2 μm syringe filter (Millipore) and imidazole (BDH) was added to 50 mM, before loading onto a 1 ml HisTrap column (GE Healthcare) using an ÄKTA Basic purification system (GE Healthcare).

During purification, buffers were either free of exogenously added lipid or supplemented with phospholipids (0.05 mg/ml (*w*/*v*) POPC or POPC/POPG/POPE in a 3:1:1 weight ratio). The column was washed with 30 column volumes of NiA buffer (50 mM Tris pH 7.4, 200 mM NaCl, 30% glycerol (*v*/*v*), 0.1% DDM, 0.5% CHAPS, 0.1% CHS (*w*/*v*), 50 mM imidazole) and the protein eluted by step gradient with buffer B (NiA containing 350 mM imidazole). The peak fractions were pooled and diluted fivefold in NT200 (50 mM Tris pH 7.4, 200 mM NaCl, 30% glycerol (*v*/*v*), 0.1% DDM, 0.5% CHAPS, 0.1% CHS (*w*/*v*), 1 mM EDTA). 0.1% DDM and 0.01% CHS (*w*/*v*) supplemented buffers were used for purification in the absence of CHAPS.

For comparison of DH5α/IPTG induction, BL21(DE3)/autoinduction with or without phospholipids supplementation, 0.5 ml of an overnight culture of cells was used to innoculate 500 ml of 2xYT or MagicMedia™ in 2.5 L polycarbonate baffled flasks. Induction of NTS1B expression in DH5α cells in 2xYT/0.2% glucose (*w*/*v*) was induced with 0.1 mM IPTG for 30 h at 20 °C, as described above. Autoinduction was performed as described above. Cells were harvested by centrifugation, snap frozen and stored at −80 °C. For purification, cells were thawed in 2× solubilisation buffer (2 ml/g cells) and homogenised. DNase I, lysozyme and the protease inhibitors were added (as above) and the sample incubated on ice for 30 min with stirring. DDM, CHAPS and CHS were added to a final concentration of 1%, 0.5% and 0.1% (*w*/*v*), respectively, and the sample stirred for 1 h. Cell debris was pelleted at 65,000*g*. The BL21(DE3) cell lysate was split into two samples: one for purification with buffers containing 0.05 mg/ml (*w*/*v*) POPC/POPG/POPE in a 3:1:1 weight ratio and one without phospholipids. The DH5α lysate was purified in the absence of phospholipids. Imadazole (50 mM) was added to the lysate before it was passed over a 5 ml HisTrap column (GE Healthcare) at a loading rate of 1.5 ml/min using an ÄKTA Basic purification system. The column was washed with 250 ml NiA and protein eluted with NiB in the presence or absence of phospholipids. The eluate was diluted fivefold with NT0 (50 mM Tris pH7.4, 0.5%, CHAPS, 0.1% CHS, 0.1% DDM (*w*/*v*), 1 mM EDTA, 30% (*v*/*v*) glycerol) and incubated with 5 ml of biotinylated neurotensin (NT) immobilized on Tetralink™ tetrameric avidin resin (Promega) overnight at 4 °C with shaking. Biotinylated NT was synthesised in-house using standard solid phase synthesis techniques and was bound to the resin as directed by the manufacturers' instructions. NTS1B was eluted using NT1 (NT0 plus 1 M NaCl). Where appropriate, buffers were supplemented with phospholipids. For gel analysis, fractions were separated using a 12% NuPAGE gel and MOPS running buffer (Invitrogen). Broad Range prestained protein marker was purchased from New England BioLabs. Total protein was assayed using *DC* Protein Assay Kit (Bio-Rad).

[^{3}H]-neurotensin binding assay

The amount of functionally active receptor was measured using a saturation radio-ligand-binding assay. Samples were diluted in assay buffer (50 mM Tris pH 7.4, 1 mM EDTA, 0.1% DDM, 0.5% CHAPS, 0.1% CHS, 0.1% BSA (*w*/*v*)) and incubated with 8.3 nM [^{3}H]-NT peptide (specific activity of 3.33 TBq/mmol, equating to 63,636 cpm/pmol, purchased from PerkinElmer) for 1 h at 4 °C. Receptor-bound ligand was separated from unbound by gel filtration using P30 Tris spin columns (Bio-Rad) and quantified by scintillation counting. The amount of active receptor was calculated assuming one binding-site per molecule and a molecular weight of 101 kDa for the fusion protein and a saturation of 90% of total sites (Fractional occupancy = [Ligand]/[Ligand] + Kd, where receptor Kd was determined as 0.9 ± 0.1 nM). Non-specific binding was assayed by competition with excess (13 μM) unlabelled peptide. The receptor was diluted so that the cpm of the total sample was between 500 and 2500 cpm, and for non-specific (competition samples) between 50 and 300 cpm. Specific binding was determined by subtracting non-specific cpm from total cpm. All samples were assayed in triplicate.

Results and discussion

Expression of NT1SB in different strains of E. coli

The over-expression of membrane proteins, especially those of eukaryotic origin, is often toxic to *E. coli*, resulting in either poor expression levels or even hampering transformation. The adapted strains C41(DE3) and C43(DE3) [28], derived from BL21(DE3), have allowed the expression of many toxic proteins, particularly membrane proteins [43]. This may be, at least in part, due to the formation of intracellular membranes during protein induction [44]. To examine whether these specifically developed strains would enhance the expression of NTS1, we compared its expression in DH5α, C41(DE3), C43(DE3) and the parental cell line, BL21(DE3).

The expression of NTS1B was examined in four cell lines under three different IPTG concentrations and in the absence of IPTG. The cells were harvested after 30 h of induction (expression did not increase significantly beyond this time point, data not shown). Expression levels were compared by assaying the amount of functional protein as determined using saturation [^{3}H]-NT binding assay (Table 1).

All cells lines show significant leaky expression (Table 1) in the absence of IPTG, despite the presence of the *lac* repressor gene within the expression plasmid. Uninduced expression of NTS1B in DH5α and C43(DE3) cells approaches that of IPTG-mediated induction. This, in part, may reflect the long period of expression and the growth suppression seen with inducing with IPTG.

The highest yield of NTS1B per L of cell culture was seen in BL21(DE3) cells induced with 0.25 mM IPTG (206 ± 32 μg/L), and show relatively high levels of expression across all IPTG concentrations tested (Table 1). Expression in C41(DE3) cells was also

Table 1
Expression of functionally active receptor in different cell types. Comparison of NTS1 expression in different cell strains induced by various concentrations of IPTG. The amount of functional receptor in detergent-solubilized sample was assayed by measuring saturation binding of [^{3}H]-NT.

Cell Type	Media	IPTG (mM)	OD_{600}	Yield (μg/L)
DH5α	2xYT	0	2.8 ± 0.2	45 ± 4
DH5α	2xYT	0.1	2 ± 0.3	75 ± 11
DH5α	2xYT	0.25	1.5 ± 0.1	33 ± 6
DH5α	2xYT	0.5	1.3 ± 0.1	25 ± 2
BL21(DE3)	2xYT	0	3.9 ± 0.2	38 ± 9
BL21(DE3)	2xYT	0.1	3.7 ± 0.3	196 ± 19
BL21(DE3)	2xYT	0.25	2.2 ± 0.2	206 ± 32
BL21(DE3)	2xYT	0.5	1.9 ± 0.3	150 ± 29
C41(DE3)	2xYT	0	4.0 ± 0.2	27 ± 4
C41(DE3)	2xYT	0.1	3.9 ± 0.1	101 ± 11
C41(DE3)	2xYT	0.25	2.2 ± 0.3	160 ± 27
C41(DE3)	2xYT	0.5	2.0 ± 0.1	89 ± 6
C43(DE3)	2xYT	0	3.8 ± 0.6	53 ± 6
C43(DE3)	2xYT	0.1	2.5 ± 0.1	69 ± 2
C43(DE3)	2xYT	0.25	1.9 ± 0.3	84 ± 8
C43(DE3)	2xYT	0.5	1.5 ± 0.1	100 ± 13
C43(DE3)	2xYT	1.0	1.6 ± 0.2	65 ± 6

Table 2
Autoinduction of NTS1 expression. Three formulations of autoinduction media were compared for expression of active NTS1 receptor. Where, O/N Express is Overnight Express™ Autoinduction System 2 [42], Magicmedia is Magicmedia™ [41] and Studier is a homemade autoinduction media [32]. Four cell types were tested for expression with MagicMedia™. Transformation of BL21(DE3) cells with pRARE2 plasmid, which encodes rare tRNAs, to give the Rosetta2 cell line, did not affect expression.

Cell Type	Media	OD_{600}	Yield (μg/L)
DH5α	Magicmedia	7.1 ± 1	178 ± 12
BL21	Magicmedia	5.6 ± 0.1	824 ± 22
C41	Magicmedia	6.5 ± 0.2	398 ± 14
C43	Magicmedia	5.2 ± 0.8	369 ± 7
Rosetta 2	Magicmedia	5.9 ± 0.4	704 ± 30
BL21	Studier	5.5 ± 0.3	650 ± 58
C41	Studier	6.2 ± 0.4	430 ± 62
BL21	O/N express	4.6 ± 0.2	505 ± 18
C41	O/N express	4.6 ± 0.4	395 ± 18

Table 3
The effect of supplementation with phospholipids on NTS1 purification. NTS1 was solubilized using detergents and purified using Ni-affinity chromatography. Ni-affinity purification buffers were supplemented with phospholipids and the amount of active receptor in the eluate was assayed. The DDM/CHS mix yields less protein due to reduced solubilisation efficiency [24].

Detergent mix	Phospholipid	Yield (μg/L culture)
DDM/CHS/CHAPS	No lipid	724 ± 28
	POPC	761 ± 39
	POPC:POPE:POPG (3:1:1)	839 ± 46
DDM/CHS	No lipid	483 ± 50
	POPC	468 ± 51
	POPC:POPE:POPG (3:1:1)	473 ± 31

relatively high, with the highest yields obtained with 0.25 mM IPTG (160 ± 27 μg/L). DH5α cells showed the poorest performance, with the highest yield of 75 μg/L at 0.1 mM IPTG, and a fall of expression beyond this level of IPTG. Thus, by changing from DH5α induction at 0.1 mM IPTG to BL21(DE3) induced with 0.25 mM IPTG, a 2.7-fold increase in active protein can be obtained.

Autoinduction of NTS1B

By careful formation of media, Studier [32] developed a reliable protocol for *lac* operon/promoter-dependent autoinduction of genes in *E. coli*. The media contains three carbon sources: glucose, glycerol and lactose. In the initial phase expression is suppressed by glucose and cells grow to a high density. When glucose is exhausted, lactose-dependent induction begins. Autoinduction has several advantages over IPTG induction: greater biomass, tight expression control, ease of scalability and reduced sample handling. Several commercial formulations based on this method are now available [41,42].

A comparison was made between several *E. coli* strains and three autoinduction media formulations: a homemade media based on that defined by Studier [32] and two commercial formulations: Overnight Express™ Autoinduction System 2 from Novagen [42] and Magicmedia™ from Invitrogen [41].

Autoinduction yielded far more receptor than IPTG-mediated induction, giving a two to fourfold increase over the optimal IPTG concentration for each cell type (Tables 1 and 2). Again, the optimal expression strain was BL21(DE3). The media formulation that gave the greatest yield of protein was Magicmedia™ with yields in BL21(DE3) cells reaching 824 ± 22 μg/L culture, compared with 650 ± 58 μg/L in homemade autoinduction media, 505 ± 18 μg/L in Overnight Express Autoinduction System 2 (a maximum of 206 ± 32 μg/L was obtained with IPTG-mediated induction (Table 1)). Supply of rare tRNAs (Rosetta2 cells) did not enhance expression over the BL21(DE3).

The cell culture densities reached at the end of the expression period were significantly higher than that in 2xYT media (Tables 1 and 2). Autoinduction allows cells to reach saturation prior to induction and the increased yields of receptor may simply reflect the increase in biomass at harvest. Allowing cells to reach a higher density prior to induction may therefore lead to increased receptor expression. However, when the cells were allowed to reach an OD_{600} of 1 in 2xYT media prior to IPTG induction, the receptor yield per L was either decreased or unchanged (data not shown and reported in [25]), suggesting that the stringent control of induction afforded by autoinduction is also an important determinant in the increase of expression.

The effect of phospholipids on the purification of NTS1B

Lipid-protein interactions may influence the folding, activity, organisation and stability of a membrane protein [45–48]. Lipids are, in general, loosely associated with membrane proteins, but some may be tightly associated, forming an integral part of the structure [49,50] that is essential for functionality [51]. During purification, membrane proteins are exposed to a vast excess of detergent and may become progressively delipidated and activity may only be restored by adding back specific lipids [52]. Supplementing buffers with phospholipids may therefore maintain the receptor in an active conformation and aid recovery of receptor inactivitated during the solubilisation process.

When NTS1B was extracted and purified using the optimal concentration of DDM/CHS/CHAPS (Table 3), the inclusion of POPC or a combination of phospholipids (POPC/POPG/POPE, 3:1:1) during Ni-affinity purification yielded larger amounts of active protein than when the lipids were not added (Table 3). However, when CHAPS was omitted and only DDM/CHS was used for solubilization, extraction and purification (Table 3), no enhancement in the yield of ligand-binding NTS1 obtained was observed. Presumably the lower levels of total detergent, or the absence of CHAPS results in less loss of activity due to less delipidation of the protein.

A medium-scale purification of NTS1 from DH5α and BL21(DE3) cells with and without phospholipids supplementation was compared. For expression in DH5α cells, a 3 L-scale expression was conducted using induction with 0.1 mM ITPG at 20 °C for 30 h and for BL21(DE3), a 2 L-scale expression was performed, using autoinduction in MagicMedia™ at 26 °C for 24 h. At harvest, DH5α cells had reached an OD_{600} 2.2 ± 0.1 and BL21(DE3) cells, 7 ± 0.8. Receptor was extracted using detergent-mediated solubilisation with DDM/CHS/CHAPS. NTS1B was purified using Ni-affinity chromatography followed by affinity purification using biotinylated NT immobilized onto avidin resin. For BL21(DE3)/autoinduction, the solubilized fraction was spit into two equal parts. One half was purified with buffers supplemented with 0.05 mg/ml POPC/POPG/POPE (3:1:1 *w*/*w* ratio) and the other in the absence of phospholipids. NTS1B from the 3 L-scale expression from DH5α cells (a larger culture size was used to allow easier comparison with the higher-expressing BL21(DE3)/autoinduction sample) was purified in the absence of phospholipids. The purification of NTS1B was followed using [^{3}H]-NT binding, total protein quantification by Lowry assay and SDS–PAGE (Table 4 and Fig. 1).

Detergent solubilisation of NTS1B yielded 100 ± 15 μg/L of culture active receptor from DH5α/IPTG induction and 797 ± 34 μg/L from BL21(DE3)/autoinduction (Table 4). Expression of receptor in DH5α/IPTG induction was slightly higher than that observed in the small-scale expression trial (Table 1, 75 ± 11 μg/L) and for BL21(DE3)/autoinduction, slightly lower (Table 1, 824 ± 22). But, overall, the expression levels are maintained when the culture size is scaled-up. The specific activity of the Ni-affinity purified fraction

Table 4
Overall comparison of NTS1 yields. Purification of NTS1B from DH5α and BL21(DE3) cells were compared. A 3 L-scale culture of NTS1B expressed in DH5α cells using IPTG induction was compared to a 2 L-scale culture of BL21(DE3) using autoinduction. The solubilized fraction from BL21(DE3) cells was split into two and the one purified in the absence of phospholipids (No PL) and the other with supplementation with a POPC/POPG/POPE mix (PPP). A two-step purification was performed: Ni-affinity (prefix Ni- in table) and ligand-affinity using streptavidin-biotinylated NT (prefix BioNT). TF is the unbound throughflow from loading step and ND not determined. The specific activity is expressed as nmol active receptor per mg total protein.

Cell type/supplement	Fraction	Active receptor (μg/L culture)	Total protein concentration (μg/ml)	Specific activity (nmol/mg)
DH5α	Solubilized	100 ± 15	16981 ± 907	0.016 ± 0.01
	Ni TF	3 ± 0.4	ND	
	Ni Eluate	78 ± 5	98 ± 13	2.8 ± 0.4
	BioNT TF	3 ± 0.4	ND	
	BioNT Eluate	59 ± 14	31 ± 8	9.6 ± 2
BL21	Solubilized	797 ± 43	26119 ± 461	0.002 ± 0.002
No PL	Ni TF	18 ± 1	ND	
	Ni Elution	631 ± 26	207 ± 24	3.3 ± 0.4
	BioNT TF	10 ± 1.7	ND	
	BioNT Eluate	395 ± 20	40 ± 9	10.7 ± 1.9
PPP	Ni TF	29 ± 1.4	ND	
	Ni Elution	740 ± 10	264 ± 15	3.3 ± 0.3
	BioNT TF	9 ± 0.3	ND	
	BioNT Eluate	464 ± 93	55 ± 2	9.2 ± 3.5

from BL21(DE3)/autoinduction, was 3.3 nmol active protein/mg total protein for both purifications, regardless of the presence of phospholipids and there is little apparent difference in the level of purity (Fig. 1). As seen in the smaller scale purifications, the overall yield of active receptor in the presence of phospholipids is higher, 740 ± 10 μg/L culture compared with 631 ± 26 μg/L (Table 4). This suggests that the presence of phospholipids in the purification buffer may aid solubilisation of the receptor, rather than stabilizing activity. The Ni-column elute from DH5α cells was slightly lower, 2.8 (±0.4) nmol/mg, but not to a significant level. An additional ligand-affinity purification step using biotinylated NT resin yields a specific activity near the maximum predicted (for NTS1B, with a molecular weight of 101 kDa, the maximum potential specific activity is 9.9 nmol/mg) for all purifications (Fig. 1 and Table 4).

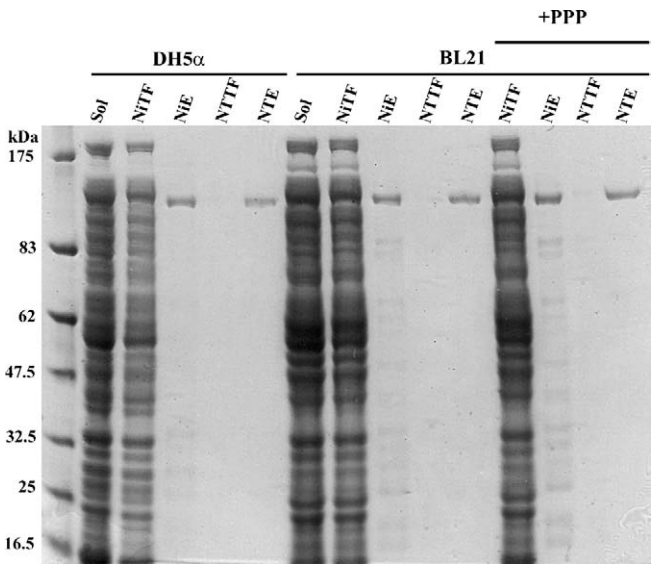

Fig. 1. Overall comparison of purification. Purification of NTS1B from DH5α/IPTG (3 L culture) induction and BL21(DE3)/autoinduction (2 L culture). The solubilized fraction from BL21(DE3) cells was split into two and one purified in the absence of phospholipids and the other with buffers supplemented with a POPC/POPG/POPE mix (+PPP). A two-step purification was performed. The solubilized (Sol) fraction was passed over a Ni-column and eluted with imidazole (NiE). Lanes labelled NiTF are the unbound fraction from column loading. The eluate was bound to biotinylated NT streptavidin-resin and eluted using a high salt buffer (NTE). Through flow from this resin is labelled NTTF. NTS1B migrates between the 175 and 83 kDa marker (molecular weight marker in the left-hand lane). The corresponding activity and protein assay data is given in Table 4.

Yields in presence of phospholipids were approximately 1.2 times greater than without. It may therefore be prudent to include phospholipids throughout other purification steps, such as the protease-mediated removal of tags and gel filtration, which may be required for some structural studies.

Conclusions

Structural biology studies generally require large (mg) amounts of a protein and where labelling is required (such as NMR), higher yields per volume of culture is a priority. Bacterial expression of heterologous proteins is often used to accomplish this. For eukaryotic membrane proteins this is particularly challenging, especially so for the GPCR-family [53]. Here we have tested potential protocol improvements in order to maximise the yield of *E. coli* expressed NTS1.

The use of alternative expression strains revealed that BL21(DE3) cells yielded an approximate 2.5-fold increase of receptor expression when compared with DH5α, which have been traditionally used for its expression [10]: 0.2 mg/L compared with 0.08 mg/L of active receptor. As with many aspects of membrane protein work no generalisation can be made and the effect of cell type on expression may be GPCR specific; the M2 Muscarinic is expressed at 1.5-fold greater levels in BL21 cells than DH5α cells [13], but the expression of the adenosine A2a receptor and CB2 cannabinoid receptor [9] showed no cell type preference. BL21(DE3) cells displayed relatively stable expression of NTS1B over a range of IPTG concentrations, as seen with adenosine A2a receptor [11]. In other the cell types tested optimizing IPTG concentration was critical for obtaining maximal NTS1B expression.

In all cell types tested, leaky expression was observed in the absence of IPTG, which may significantly hamper expression. Autoinduction, a method that relies on the switch from catabolite repression to substrate induction, has been shown to improve the expression of heterologous proteins in *E. coli* [32,33]. Compared with even the highest levels of IPTG-mediated induction, autoinduction gave vastly improved expression levels: up to 0.8 mg/L of cell culture [53]. Unlike cell type and IPTG concentration, it is likely that this will be true for most GPCRs expressed in *E. coli*, allowing cell high densities without the detrimental effects of leaky expression.

The inclusion of phospholipids during the purification process enhanced the recovery of active receptor. A combination of POPC/POPG/POPE (3:1:1) gave a 14% increase in the amount of

active protein following Ni-affinity purification when compared to the omission of lipids. The enhanced effects of added lipids were only evident with DDM/CHS/CHAPS solubilized receptor. When only DDM/CHS was present no enhancement was observed, suggesting that delipidation was the main factor. The specific activity of the receptor is similar in the presence or absence of lipids. Therefore, lipid does not appear to increase the proportion of active receptor. The enhanced yield may therefore reflect the enhanced solubilisation of receptor, either by masking hydrophobic regions or preventing denaturation. As the lipid mixture was more effective than POPC alone, there may also be a lipid specific component. *E. coli* membranes and eukaryotic membranes differ significantly in composition; *E. coli* lacks phosphatidylserine and phosphatidylcholine which together make up >20% of brain lipids [54].

Producing active GPCRs in *E. coli* has proved challenging, particularly in terms of achieving the yields required for input into structural biology. Autoinduction is a simple expression method, made even easier by the availability of commercial formulations and can be adapted for labeling protocols [42,55]. Indeed, autoinduction has several advantages, such as reduced sample handling and when coupled to screening techniques such as fluorescent protein labelling of GPCRs, which we have demonstrated for NTS1 [56], could be a great benefit for low volume, high-throughput screening of GPCR expression in *E. coli* [31].

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