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Overexpression of membrane proteins in mammalian cells for structural studies

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Abstract

The number of structures of integral membrane proteins from higher eukaryotes is steadily increasing due to a number of innovative protein engineering and crystallization strategies devised over the last few years. However, it is sobering to reflect that these structures represent only a tiny proportion of the total number of membrane proteins encoded by a mammalian genome. In addition, the structures determined to date are of the most tractable membrane proteins, i.e., those that are expressed functionally and to high levels in yeast or in insect cells using the baculovirus expression system. However, some membrane proteins that are expressed inefficiently in these systems can be produced at sufficiently high levels in mammalian cells to allow structure determination. Mammalian expression systems are an under-used resource in structural biology and represent an effective way to produce fully functional membrane proteins for structural studies. This review will discuss examples of vertebrate membrane protein overexpression in mammalian cells using a variety of viral, constitutive or inducible expression systems.

Keywords: *Membrane protein structure, inducible mammalian cell expression systems, overexpression*

Introduction

Heterologous overexpression of integral membrane proteins in bacteria, yeasts and insect cells (Grisshammer and Tate 1995, Midgett and Madden 2007) has allowed the structure determination of over 300 integral membrane proteins from diverse families and from a wide variety of organisms from archaebacteria to man (Vinothkumar and Henderson 2010). However, only about 10% of the unique membrane protein structures determined are derived from vertebrates. There are two reasons for this. Firstly, many eukaryotic membrane proteins are unstable during detergent solubilization and purification (Tate 2010), although generic strategies have recently been devised to tackle this problem (Bill et al. 2011). Secondly, overexpression of eukaryotic membrane proteins can be problematic, often with poor expression of functional protein in the most commonly used expression systems (bacteria, yeasts, insect cells) (Tate 2001). Recently, structures of mammalian membrane proteins have been determined after overexpression in mammalian cells (Table I); both bovine rhodopsin (Standfuss et al. 2007, Standfuss et al. 2011, Deupi et al. 2012) and the human ammonia transporter RhCG (Gruswitz et al. 2010) were most effectively produced in a fully folded state in these cells. This shows it is possible to produce milligrams of a membrane protein in mammalian cells suitable for crystallization.

The reasons why some membrane proteins are overexpressed easily whilst others are expressed poorly are not fully understood (Grisshammer and Tate 1995, Grisshammer 2006). However, what is clear is that this problem is not proportional to the number of transmembrane α-helices or the size of the protein, but is related to the 'complexity' of the membrane protein, i.e., how difficult it is to fold into a functional state (Tate 2001). It is likely that the level of functional membrane protein expression is dictated by a complex interplay of factors that probably include the following: The amount of mRNA synthesized and its stability, the secondary structure of the mRNA and the presence of translational pause sites, folding of the nascent polypeptide chain in the ribosome and translocon, the efficiency of insertion into the membrane, the role of post-translational modifications, e.g. N-glycosylation, in the folding process and the requirement for molecular chaperones to facilitate folding.

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Table I. Structures of mammalian membrane proteins determined from protein overexpressed in mammalian cells.

Membrane protein	Source	Cells used for protein production	Resolution of structure*	Reference
Ammonia transporter RhCG	Human	HEK293S(TetR)-GnTI⁻	2.1 Å	(Gruswitz et al. 2010)
Rhodopsin N2C/D282C/D113Q	Bovine	HEK293S(TetR)-GnTI⁻	3.0 Å	(Standfuss et al. 2011)
Rhodopsin N2C/D282C/M257Y	Bovine	HEK293S(TetR)-GnTI⁻	3.3 Å	(Deupi et al. 2012)
Rhodopsin N2C/D282C	Bovine	COS-7	3.4 Å	(Standfuss et al. 2007)
Connexin a1 (gap junction)	Human	BHK	7.5 Å	(Unger et al. 1999)

*Structures were determined by X-ray diffraction except for the structure of connexin, which was determined by electron cryo-microscopy of 2-dimensional crystals that formed in BHK cells upon overexpression. There are numerous examples of low resolution structures determined by electron microscopy of membrane proteins isolated after expression in mammalian cells, but only examples of structures at better than 8 Å resolution are shown. Details of the expression systems and amounts of protein expressed are shown in Tables II and III.

These factors vary considerably between different expression systems. It is not possible to predict which expression system will be the best for any particular membrane protein, but it has been found that it is easier to overexpress functional mammalian membrane proteins in insect cells rather than in *Escherchia coli*, presumably because the insect cells have evolved to fold membrane proteins similar to their mammalian counterparts. Even within a single expression system it is not possible to predict the expression levels of a membrane protein just by analyzing its amino acid sequence, but it has been found that even single point mutations can significantly improve the expression of membrane proteins (Magnani et al. 2008, Serrano-Vega et al. 2008, Warne et al. 2009).

The inability to predict expression levels of a membrane protein from its amino acid sequence has meant that the most pragmatic strategy to overexpress membrane proteins is based on prior experience, i.e., if the baculovirus expression system has worked for one particular membrane protein, it may be a good choice for other similar proteins. To overcome the disparities in expression levels due to different amino acid sequences and/or mRNA structure, the expression of a variety of homologues from different species are tested and the highest expressers are then chosen for further characterization (Mancia and Love 2010, 2011). Expression can be improved further either through co-expression of molecular chaperones (Tate et al. 1999, Higgins et al. 2003, Zhang et al. 2003) or through the introduction of specific point mutations that increase expression levels (Magnani et al. 2008, Shibata et al. 2009). This review will focus specifically on mammalian expression systems and why they may have significant advantages over other expression systems for the overexpression of some eukaryotic membrane proteins.

Why use a mammalian expression system?

The choice of which expression system to use for a new target is usually dictated initially by which expression systems are already in use in the laboratory or in adjacent laboratories. This increases the speed at which results are obtained, because user-knowledge can greatly improve the yield from a particular expression system, especially when using insect cells or mammalian cells where the health of the cells before production is vital to obtain good yields. However, in a few instances it may be essential to use mammalian cells because of the characteristics of a particular membrane protein. The most detailed comparison between different expression systems performed on a single membrane protein is for the serotonin transporter (SERT) (Tate et al. 2003), which has a number of properties that make it particularly challenging for overexpression. Firstly, there are two N-glycosylation sites in extracellular loop 2 that are essential for efficient folding of the protein (Tate and Blakely 1994). Mutation of both the N-linked Asn to Gln reduced functional expression in the baculovirus expression system by 20-fold, although the K_m for transport and the K_D for inhibitor binding were unaffected (Tate and Blakely 1994). All mammalian homologues of SERT are N-glycosylated in this region and, of those tested, all show similar results to SERT (Melikian et al. 1996, Straumann et al. 2006). Secondly, SERT appears to require the molecular chaperone calnexin for efficient folding (Tate et al. 1999). This is probably related to the requirement for N-glycosylation, because calnexin binds specifically to glucosylated forms of N-glycans as part of the quality control system in the endoplasmic reticulum (Ellgaard and Helenius 2003, Helenius and Aebi 2004). Thirdly, SERT has a strict requirement for cholesterol (Scanlon et al. 2001), presumably for stabilising the folding state of the transporter. Removal of cholesterol from the membrane abolished transport activity and inhibitor binding, which could be recovered upon addition of exogenous cholesterol, but not with cholesterol analogues. Finally, SERT is oligomeric (Kilic and Rudnick 2000), although how this impacts upon expression levels is unknown.

Table II. Expression of membrane proteins using transient expression systems in mammalian cells.

Membrane protein	Promoter	Cell line	Scale up	Expression levels*	Amount purified	Reference
Semliki Forest Virus expression						
Neurokinin 1 receptor	SFV subgenomic	BHK-21	–	40 pmol/mg	–	(Lundstrom et al. 1994)
Histamine receptor H2	SFV subgenomic	COS-7	–	80 pmol/mg	–	(Hoffmann et al. 2001)
α2β adrenergic receptor	SFV subgenomic	CHO	–	175 pmol/mg mp; 8.8 million c/c	–	(Sen et al. 2003)
Adenosine receptor A2A	SFV subgenomic	BHK-21	–	285 pmol/mg; 1–10 mg/l	–	(Hassaine et al. 2006)
Glutamate transporter GLT1	SFV subgenomic	BHK-21	Stirrer flasks (8 l) 1 million cells/ml	3.5 million c/c; 61 pmol/mg mp	0.3 mg	(Raunser et al. 2005)
Bradykinin B2 receptor	SFV subgenomic	BHK-21	–	11 pmol/mg; 0.2 mg/l	–	(Shukla et al. 2006a)
Aquaporin AQP3	SFV subgenomic	various	1 l glass roller bottles	~ 0.01 mg/l	–	(Eifler et al. 2007)
Aquaporin AQP6	SFV subgenomic	various	–	<0.001 mg/l	–	(Eifler et al. 2007)
HCN2 channel	SFV subgenomic	various	–	~ 10 mg/l (+ misfolded)	–	(Eifler et al. 2007)
Purine receptor P2X2	SFV subgenomic	various	–	~0.5 mg/l	–	(Eifler et al. 2007)
Purine receptor P2Y2	SFV subgenomic	various	–	~ 10 mg/l	–	(Eifler et al. 2007)
Vasopressin receptor V2	SFV subgenomic	various	–	~ 1 mg/l	–	(Eifler et al. 2007)
Angiotensin receptor AT1	SFV subgenomic	BHK	–	32 pmol/mg	–	(Shukla et al. 2006b)
Muscarinic M3 receptor	SFV subgenomic	COS-7	–	27 pmol/mg	–	(Romero-Fernandez et al. 2011)
Adenovirus virus expression						
TRH receptor	CMV	HeLa	–	2 million c/c	–	(Falck-Pedersen et al. 1994)
Vaccinia virus expression						
Neuropeptide Y receptor	Vaccinia 11K promoter	HeLa	–	1 mg/l	–	(Walker et al. 1993)
Transient transfection from plasmid DNA						
β2 adrenergic receptor	Adenovirus major late promoter	COS-1	–	18 pmol/mg	–	(Chelikani et al. 2006)
Rhodopsin mutant		COS-7	50 × 15 cm plates	n.r.	~ 0.6 mg	(Standfuss et al. 2007)

*Shown are some examples of membrane proteins expressed in mammalian cells at high-levels or where there are interesting comparisons between expression systems. Further examples can be found in (Grisshammer and Tate 1995, Sarramegna et al. 2003). Expression levels are shown in units as supplied in the primary publication: c/c, copies per cell; mg mp, milligram of membrane protein; n.r., not recorded.

In the comparison of expression systems for SERT, clearly *E. coli* and yeasts were inappropriate given the requirement of cholesterol for activity (absent in both organisms) and for N-glycosylation for efficient folding (absent in *E. coli*). Insect cells contain cholesterol, they can N-glycosylate proteins and they contain calnexin, so, based on the effectiveness of the system for producing many mammalian membrane proteins it would be expected that it would also be suitable for SERT. Indeed, functional SERT was detected using both transport assays and inhibitor binding, but it was apparent from comparative western blotting that all the unglycosylated SERT produced (over 99% of the total SERT, depending on the cell type) was inactive (Tate and Blakely 1994) and also over 90% of the glycosylated SERT was also inactive (Tate et al. 1999). Co-expression of calnexin did improve the overall expression levels of functional SERT by 3-fold, but did not prevent the formation of inactive transporter (Tate et al. 1999). It was at this juncture that mammalian expression systems were tried (Tate et al. 2003) and it was found that a tetracycline-inducible system was the most effective for producing fully functional SERT for structural studies (see below).

The choice of whether to use a mammalian expression system or whether to use the baculovirus expression system (or indeed yeasts) can only be made in the presence of biochemical data or on expression data. We would certainly recommend trying mammalian cells if the baculovirus expression system yields low levels of functional protein and/or large quantities of misfolded, inactive protein. The near-native environment will virtually guarantee functional expression (except if the target is a hetero-oligomer or requires cell-specific folding factors) since it can provide correct N-glycosylation, post-translational machinery and molecular chaperones and a suitable lipid environment containing cholesterol. In addition, we would consider making stable cell lines as a matter of course for any mammalian membrane protein, because they provide an excellent resource as a positive control in biochemical assays and for comparison with other expression systems.

Choice of mammalian expression system

When choosing a mammalian expression system to overexpress membrane proteins there are a number of decisions required. The first choice is whether to express the membrane protein using a transient system or whether to make stable cell lines. The use of transient expression systems allows protein production within a few days after the expression plasmid has been constructed, whereas stable cell lines take months to develop. Transient expression systems may rely on using either cationic compounds to facilitate the uptake of plasmid DNA into the cell or recombinant non-replicative viruses that have evolved to efficiently enter mammalian cells. For both transient expression and the construction of stable cell lines there is the choice of whether to use an inducible promoter or whether to use a constitutive promoter. Finally, the cell line to be used for the expression studies needs to be determined. Although this implies that there is a considerable diversity of choice, in actual fact only a limited selection of expression systems have been systematically studied and there are very few examples in the literature of large-scale expression of vertebrate membrane proteins (Tables II and III). We have therefore included in this section the most commonly used systems, with only passing mention of other systems that could be used; for a more exhaustive analysis of mammalian expression systems, see (Fussenegger 2001).

Transient transfection using recombinant viruses

The attraction of expressing proteins using recombinant viruses is that generally the viruses are very efficient at entering a cell and they usually have very strong promoters from which proteins can be expressed. Therefore, once the recombinant virus has been constructed, membrane protein expression is a reproducible, efficient process even on a large scale; the success and popularity of the baculovirus expression system is an excellent example (Jarvis 2009). However, the safety of handling recombinant viruses is an important consideration. Recombinant baculoviruses can infect mammalian cells (Hofmann et al. 1995, Boyce and Bucher 1996), but they cannot replicate in them and the polyhedrin promoter does not express the membrane protein of interest. However, if the polyhedrin promoter is replaced by a CMV promoter that is functional in mammalian cells, then the membrane protein will be expressed (Kost et al. 2005, Dukkipati et al. 2008). Baculovirus-mediated expression using a mammalian promoter is generally considered safe, because the recombinant baculovirus cannot replicate in mammalian cells. However, viruses that have evolved naturally to infect mammalian cells can obviously replicate, causing infections and disease. Therefore viruses that are used in the laboratory to infect mammalian cells are usually extensively disabled to make them safer to work with. For example, some expression systems use virus-like particles (VLPs) that are produced in a packaging cell line so that they contain only the essential requirements for expressing the gene of interest and they are thus incapable of replication.

Table III. Expression of membrane proteins in stable mammalian cell lines.

Membrane protein	Promoter	Cell line	Scale up	Expression levels*	Amount purified	Ref
Constitutive expression in stable mammalian cell lines						
β2 adrenergic receptor	CMV	CHO	—	200 pmol/mg	1.3 nmol (~60 ug)	(Lohse 1992)
Glucagon receptor	CMV	BHK	—	78 pmol/mg	—	(Jelinek et al. 1993)
Serotonin transporter	CMV	293EBNA	—	290,000 c/c	—	(Tate et al. 2003)
Chemokine receptor D6	CMV	L1.2	1 mg/5 × 10^9 cells	2.5 million c/c	—	(Blackburn et al. 2004)
Serotonin receptor 5HT2c	CMV	HEK293T	—	140 pmol/mg; 3 million c/c	—	(Mancia et al. 2004)
Bradykinin receptor B2	CMV	HEK293S	—	3 million c/c; 60 pmol/mg mp	—	(Camponova et al. 2007)
Chemokine receptor CCR1	CMV	Flp-In 293	—	Relative fluorescence	—	(Allen et al. 2009)
Inducible expression in stable mammalian cell lines						
Serotonin transporter	Sindbis virus subgenomic	BHK-21	Suspension culture: 0.5 million cells/ml	240,000 c/c	—	(Tate et al. 2003)
Serotonin transporter	β-globin	MEL	Suspension culture: 1 million cells/ml	80,000 c/c	—	(Tate et al. 2003)
Serotonin transporter	CMV/TetO2(pcDNA4)	T-REx 293	—	400,000 c/c	—	(Tate et al. 2003)
Chemokine receptor CCR1	CMV/TetO2(pcDNA4)	T-REx 293	—	Relative fluorescence	—	(Allen et al. 2009)
Olfactory receptor 17-4	CMV/TetO2(pcDNA4)	HEK293S-TetR	50 × 15 cm plates	~ 30 ug/15 cm plate	0.13 mg (50 plates)	(Cook et al. 2008)
Olfactory receptor 17-4	CMV/TetO2(pcDNA4)	HEK293S-GnTI	Bioreactor (1.25 l): 9.6 million cells/ml	-	3 mg per litre	(Cook et al. 2009)
GABA$_A$ receptor α1β3	CMV/TetO2(pcDNA4)	HEK293S-TetR	Bioreactor 3 l	3.2 nmol/l; 14 pmol/mg	1.4 mg	(Dostalova et al. 2010)
Serotonin receptor 5HT3A	CMV/TetO2(pcDNA4)	HEK293S-TetR	Bioreactor 3 l	9-34 nmol/l; 24-47 pmol/mg	5.5 mg	(Dostalova et al. 2010)
Serotonin transporter	CMV/TetO2(pcDNA4)	T-REx 293	3.9 × 10^8 cells from tissue culture plates	1.22-1.67 million c/c	0.59 nmol (~50 ug)	(Takayama and Sugio 2011)
Vomeronasal receptor 1	CMV/TetO2(pcDNA4)	HEK293S-GnTI	Tissue culture plates (unknown quantity)	n.r	1 mg/g of cells	(Corin et al. 2011)
Chemokine receptor CCR1	CMV/TetO2	Flp-In T-REx 293	—	Relative fluorescence only	—	(Allen et al. 2009)
Rhodopsin	CMV/TetO2(pACMV)	HEK293S-TetR	Bioreactor (5.5 l): 5 million cells/ml	9 mg/l	—	(Reeves et al. 2002b)
Rhodopsin	CMV/TetO2(pACMV)	HEK293S-GnTI	Bioreactor (1.1 l): 4 million cells/ml	6 mg/l	—	(Reeves et al. 2002a)
β2 adrenergic receptor	CMV/TetO2(pACMV)	HEK293S-TetR	Tissue culture plates	220 pmol/mg mp	12 ug/15 cm plate	(Chelikani et al. 2006)
Bradykinin receptor B2	CMV/TetO2(pACMV)	HEK293S-TetR	—	100 pmol/mg mp; 5.5 million c/c	—	(Camponova et al. 2007)
Chemokine receptor CCR1	CMV/TetO2(pACMV)	HEK293S-TetR	CellStack or spinner flasks	n.r.	0.1-0.2 mg/ 10^9 cells	(Allen et al. 2009)
Tetraspanin CD81	CMV/TetO2(pACMV)	HEK293S-TetR	15 cm tissue culture plates	~ 26 ug/30 million cells	—	(Takayama et al. 2008)
Glucagon receptor	CMV/TetO2(pACMV)	HEK293S-GnTI	10 cm plates	1.2 ug/mg mp	—	(Unson 2008)
Rhodopsin mutants	CMV/TetO2(pACMV)	HEK293S-GnTI	Wave bioreactor 10 l	n.r	~0.5 mg per litre**	(Standfuss et al. 2011, Deupi et al. 2012)
Ammonia transporter RhCG	CMV/TetO2(pACMV)	HEK293S-GnTI	Wave bioreactor: 1 million cells/ml	n.r.	0.5 mg per litre	(Gruswitz et al. 2010, Chaudhary et al. 2012)

*Shown are some examples of membrane proteins expressed in mammalian cells at high-levels or where there are interesting comparisons between expression systems. Further examples can be found in (Grisshammer and Tate 1995, Sarramegna et al. 2003). Expression levels are shown in units as supplied in the primary publication: c/c, copies per cell; mg mp, milligram of membrane protein; n.r., not reported.
**Personal communication.

Although many such systems have been developed based on, for example, lentiviruses (Cockrell and Kafri 2007, Matrai et al. 2010) and adenovirus (Russell 2000), they have been used predominantly for gene therapy and cell biological experiments, and they have not been tested for the overexpression of membrane proteins in mammalian cells. However, the Semliki Forest Virus (SFV) expression system (Liljestrom and Garoff 1991, Berglund et al. 1993) has been used extensively to overexpress integral membrane proteins, in particular G protein-coupled receptors (Hassaine et al. 2006) and this will be the focus for the remainder of this section.

Recombinant SFV is generated in BHK-21 cells by co-electroporating recombinant RNA (generated *in vitro* by transcription from a SFV expression vector that contains the SFV 26S promoter, target gene and SFV non-structural genes) with helper RNA carrying the SFV capsid and envelope genes. Since the helper RNA lacks a packaging signal, VLPs generated will only carry the recombinant RNA (Liljestrom and Garoff 1991); hence the VLPs are replication-incompetent as they lack the genes coding for the structural components of the virus. Recombinant SFV is harvested from the BHK-21 cells and activated by α-chymotrypsin prior to infecting host cells, e.g., BHK-21 or HEK293 cells grown adherently or in suspension. Optimum recombinant protein production occurs in 24–72 hours, before the cytotoxic effects of the SFV infection kill the host cells (Liljestrom and Garoff 1991).

The SFV expression system has been used to express successfully a wide variety of vertebrate membrane proteins. In one study, 100 GPCRs were expressed and, where binding assays were available, many of the GPCRs were shown to be functional (Hassaine et al. 2006). In another example, the rat glutamate transporter GLT1 was expressed at ~ 0.3 mg/l, which allowed its purification and the determination of a low resolution structure by single-particle electron microscopy (Raunser et al. 2005). However, although expression of membrane proteins is invariably successful, there appears to be a significant problem due to the retention of a large proportion of the expressed polypeptide in the ER, which often correlates with this population of the protein being misfolded. Indeed, where experiments have been performed to look at the functionality of the expressed membrane protein, often only a small percentage of the protein is functional. For example, high levels of intracellular retention were observed for SFV-expressed α_2 adrenergic receptor (Sen et al. 2003), the bradykinin B_2 receptor (Shukla et al. 2006a) and the angiotensin II receptor (Shukla et al. 2006b). Only 0.5% and 7% of the ion channels $P2X_2$ and HCN2, respectively, were located in the plasma membrane after expression using SFV (Eifler et al. 2007). This problem was also observed for GPCRs; the SFV-expressed vasopressin receptor, V_2R, was virtually entirely intracellular when expressed in BHK-21 cells, with only 0.005% of the total recombinant protein being active as defined by ligand-binding assays (Eifler et al. 2007). However, expressing V_2R in HEK293 cells increased the proportion of active protein to 20% with higher expression observed at the plasma membrane (Eifler et al. 2007). It was noticeable in the comparison of expression of 101 GPCRs using the SFV expression system that there was no correlation between the western blot signal for the GPCR and its functionality as assessed by ligand binding (Lundstrom et al. 2006). This suggests that there was considerable variation in the percentage of each receptor that was actually expressed in a functional form. To date, no GPCR structures have been determined from receptors expressed using the SFV system.

The SFV expression system has a number of serious drawbacks. It is expensive and technically challenging to make large amounts of RNA to make sufficient recombinant virus for large-scale expression studies, although it is fast and efficient for small-scale pilot studies. In addition, although recombinant SFV is highly disabled, many countries consider it should be used at biosafety level 2, which makes large-scale cultures more onerous to produce. Results from numerous studies have shown that a considerable proportion of the expressed membrane protein is misfolded. In combination, these factors have meant that currently the SFV expression system is not widely used.

Transient transfection using chemical reagents

Transient transfection of plasmid DNA into mammalian cells is an efficient and cost-effective method for expressing membrane proteins, even on a large scale (Geisse 2009, Geisse and Fux 2009). The method is often used on an analytical scale to assess the effect of mutations and truncations on membrane proteins prior to scale-up for purification in either mammalian or insect cell expression systems. However, it has also been developed to express sufficient protein for purification, crystallization and structure determination (Aricescu et al. 2006). Essentially any mammalian expression plasmid optimized for protein expression can be used in combination with any cell line. However, the efficiency of transfection decreases with very large plasmids and some cell lines may be refractory to transfection. Improvements in transfection efficiency may be obtained using commercial preparations of cationic lipids and/or using electroporation, but these systems are often too expensive or not amenable to scale-up.

Factors that affect the level of expression obtained for a membrane protein in transiently transfected cells include the plasmid size, the amount of plasmid used per transfection, the strength of the promoter, the cell type, the efficiency of the transfection and potentially the toxicity of the transfection reagent. GFP is a convenient marker protein to allow rapid optimization of transfection conditions. Membrane protein expression can be conveniently followed either by using GFP fusions, western blotting or ligand binding assays. It is worth mentioning that the ratio of plasmid DNA added per reaction may be an important factor in obtaining functional expression of a membrane protein in the plasma membrane, particularly if a strong CMV promoter is being used. If too much plasmid is used, all the expressed protein may reside intracellularly and could be misfolded. In these instances, we have found that using a weaker promoter or an inducible promoter (see below) may be beneficial.

The most successful example of using transient transfection for the expression of membrane proteins is the production of a thermostable rhodopsin mutant for structure determination (Standfuss et al. 2007). Bovine rhodopsin was expressed in COS-7 cells from a constitutive adenovirus promoter after transient transfection using DEAE dextran that resulted in a total of 2.5 mg rhodopsin from 50 transfected 15 cm plates, which yielded ~ 0.6 mg of pure protein for crystallization trials. The protein was subsequently crystallized and the structure determined to 3.4 Å resolution (Standfuss et al. 2007).

The major advantage of transient protein expression is that it is quick, because it takes only a few days to go from purified plasmid DNA to expressed protein. This can be a considerable asset in structural biology where a number of different mutations may need to be tried to improve the diffraction quality of crystals. However, when scaling up transient transfections to litre volumes there is often batch-to-batch variability in the amount of protein expressed. This may not be an issue if the normal levels of expression are high, as is the case for bovine rhodopsin.

Construction of stable cell lines

In contrast to transient expression, making stably transfected cell lines is a lengthy process that usually requires the stable integration of the recombinant DNA into the host cell genome. Since the expression vector carries an antibiotic resistance gene, stable integrants can be identified by an antibiotic selection, which typically takes several weeks. Integration of the transgene into the host cell genome may either be random or the host cell may be engineered to contain a specific sequence recognized by a recombinase that allows targeted integration. If random integration is used, then the expression levels obtained are strongly dependent on where the transgene integrates. Selection of clonal cells is then required to identify highly expressing cell lines that are stable under prolonged culture. Clonal selection is typically made through limited dilution where recently transfected cells are serially diluted and seeded on tissue culture plates with antibiotic-containing media. Colonies of cells appear in 3–6 weeks, at which point they are individually transferred to 24-well plates and scaled up. Expression levels from each cell line may then be assessed by western blotting and/or radioligand binding assays. Another approach to select for highly expressing cells is to use fluorescence-activated cell sorting (FACS), usually through the use of GFP. For example, Mancia et al. (2004) linked the expression of the 5HT2c serotonin receptor to the expression of eGFP by inserting an internal ribosomal entry site (IRES) in between the two genes. Hence high levels of fluorescence were indicative of high levels of expression of the 5HT2c serotonin receptor. FACS was used to sort the highly fluorescent cells iteratively to produce cell lines expressing 5HT2c serotonin receptor at 3 million copies/cell (Mancia et al. 2004).

The major decision to be made before making a stable cell line is whether to use a constitutive promoter or an inducible promoter to express the membrane protein of interest. From the limited data we currently have, it appears that there are significant problems associated with using strong constitutive promoters for the production of membrane proteins due to the loss of expression upon prolonged cell growth, which is of course essential when they need to be grown on a litre scale. For example, stable cell lines that constitutively expressed SERT grew very slowly and only about 25% of the cells actually expressed the transporter, which precluded large-scale culture (Tate et al. 2003). In another example, expression levels of the bradykinin B_2 receptor decreased with each passage, until expression levels reached half its original value, after which no further reduction was observed (Camponova et al. 2007). Similarly, high-level expression of the serotonin 5HT2c receptor was only maintained by routine FACS sorting of the most highly expressing cells (Mancia et al. 2004). The slow growth rate, loss of expression over multiple cell passages and the relatively low expression levels all indicate that the cells are severely stressed. This is likely due to the high metabolic demands placed on the cells when overexpressing a membrane protein and/or on the adverse biological activity of the membrane protein on the cells.

In order to circumvent the negative effects of constitutive membrane protein overexpression on cell growth, an inducible system can be used. In an inducible system the expression can be switched on or off by changing an external factor, such as temperature or the addition of a chemical, which is analogous to many bacterial expression systems. Hence protein expression is limited during large-scale cell growth, allowing mammalian cell cultures to reach the desired high cell density. At this point, expression is switched on for a short period of time, typically 24–72 h prior to harvesting of the cells. Comparative studies between constitutive and inducible expression of various membrane proteins such as rhodopsin (Reeves et al. 1996, 2002b), SERT (Tate et al. 2003), the β_2-adrenergic receptor (Chelikani et al. 2006) and the bradykinin B_2 receptor (Camponova et al. 2007) have shown that a tetracycline inducible expression system can produce 4- to 12-fold more membrane protein than the constitutive counterpart (Table III) and, more importantly, the cells grow robustly and are capable of large scale culture.

Inducible mammalian cell expression systems

Development of inducible mammalian expression systems has been driven predominantly by the requirements of cell biologists who want to study the effects of specific gene products in living systems. In these experiments, it is ideal to have no expressed protein in the uninduced state and, upon induction, physiological levels of the desired protein are synthesized. Hence the expression systems are generally characterized as having very weak promoters that are reasonably tightly regulated, such as the tetracycline on/off (tTA and rtTA) systems developed by Bujard and colleagues (Gossen and Bujard 1992, Kistner et al. 1996, Baron and Bujard 2000). Other tightly controlled systems have also been developed, including systems induced either by ecdysone (No et al. 1996), cumate (Mullick et al. 2006), temperature (Boorsma et al. 2000) or DMSO (Needham et al. 1995). In addition, an alternative tetracycline-inducible system was also developed (Yao et al. 1998) that uses the strong CMV promoter, which is repressed by having tandem tetracycline operator sites (TetO) between the promoter and the start of the gene of interest and using cell lines that constitutively express the tetracycline repressor protein TetR. Of these expression systems, only three have been directly compared for membrane protein production: Namely the tetracycline-inducible system (Yao et al. 1998), the cold-inducible system (pCytTS; Cytos (Boorsma et al. 2000) and the DMSO-inducible system (induction of differentiation of

mouse erythroleukaemic cells (Needham et al. 1995). In this comparative study of SERT expression (Tate et al. 2003), the tetracycline-inducible system was the most successful in terms of the amount of transporter expressed and the lack of unglycosylated, misfolded material in the endoplasmic reticulum, which is the characteristic problem observed for this transporter using the baculovirus expression system. Interestingly, the most efficient cell line for producing polypeptide was the cold-inducible pCytTS system, but in the case of SERT, the majority of this polypeptide was unglycosylated and misfolded; other membrane proteins may be expressed in a fully functional state to high levels using this system. As the tetracycline-inducible system was the most successful of the expression systems tested and it has also now been used for multiple different membrane proteins (Table III), it will be discussed in more detail below.

In the tetracycline-inducible expression system (Yao et al. 1998) the expression vector includes tandem Tet operator sites (TetO2) located close to and downstream from the promoter region. The repressor protein TetR binds to TetO, thus sterically preventing transcription from occurring. Addition of tetracycline to the media inactivates TetR, which releases it from binding the operator sequences and allows transcription from the CMV promoter to proceed. The tetracycline-inducible expression system can be used as a transient expression system, where TetR and the gene of interest are transiently transfected into cells from purified plasmids (Yao et al. 1998). Alternatively, stable cell lines can be developed based on mammalian cells engineered to constitutively expresses TetR, which initially included Vero and U2OS cell lines (Yao et al. 1998). This latter system was popularized by the development of a HEK293S-TetR cell line (Reeves et al. 2002b), which was used to overexpress a mutant of rhodopsin that failed to express in a constitutive expression system, presumably due to its toxicity to the cells (Reeves et al. 2002b). The tetracycline-inducible system of Yao et al. (1998), commercialized as the T-Rex system (Invitrogen), was shown to be the most effective system for the production of SERT (Tate et al. 2003) and it has now been used on many other membrane proteins (Table III). In the case of the ammonia transporter RhCG (Chaudhary et al. 2012), the tetracycline-inducible system allowed its purification, crystallization and structure determination (Gruswitz et al. 2010). Expression using the tetracycline-inducible system is characterized by predominantly cell surface expression, which is indicative of correctly folded membrane protein (Ellgaard and Helenius 2003). The ability to grow the cells in the uninduced state means that the cell lines grow with a similar rate to the

parental cell line, allowing large-scale preparations. The advantage of having a stable cell line is that expression levels between cell batches is very similar. The major disadvantage is the time required to make good stable cell lines, especially if multiple constructs have too be made to facilitate the production of well-diffracting crystals.

Cell line selection

All mammalian cell lines are anticipated to provide a near-native environment for overexpressed mammalian membrane proteins, with correct post-translational modifications and lipid environment. Cell lines predominantly used in the context of mammalian membrane protein expression (Tables II and III) are human embryonic kidney cells (HEK293), baby hamster kidney cells (BHK-21), monkey kidney fibroblast cells (COS-7) and Chinese hamster ovary cells (CHO). If choosing a tetracycline-inducible system the cell line needs to stably and constitutively express TetR at appropriate levels, such as found in the HEK293S-TetR (Reeves et al. 2002b) or T-Rex 293 (Invitrogen) cell lines.

Expression levels of recombinant mammalian membrane proteins in mammalian cells are typically modest (Tables II and III), but sometimes this can been improved by the inclusion of sodium butyrate, a histone deacetylase inhibitor, which was essential to achieve very high levels of rhodopsin overexpression (Reeves et al. 2002a, 2002b). It is also important to consider how to scale-up cell production to get sufficient starting material for purification and crystallisation. Most laboratories will be able to produce 10 l of suspension culture using a wave bioreactor system (Singh 1999), as this does not require large capital investment or technological know-how, unlike traditional large bioreactors. Wave bioreactors were used to produce sufficient material for the crystallization of rhodopsin mutants (Standfuss et al. 2011, Deupi et al. 2012) and RhCG (Chaudhary et al. 2012), whereas a specialized bioreactor was used for labeling rhodopsin for NMR studies (see, for example, Ahuja et al. 2009).

One other important factor to consider when choosing a mammalian cell line is the homogeniety of the final purified protein. In some instances, N-glycosylation may be required for efficient mammalian membrane protein expression, but this produces an extremely heterogeneous protein population, often containing 10–20 kDa of flexible sugar chains. This is usually highly detrimental to successful crystallisation. In order to circumvent this problem, N-glycosylation defective mutants of cell lines are available, both for CHO (Stanley et al. 1975) and HEK293 (Reeves et al. 2002a) cells. A mutant HEK293S cell line lacking the

N-acetylglucosaminetransferase I (GnTI) enzyme was developed, which resulted in proteins homogeneously N-glycosylated with a $GlcNAc_2Man_5$ sugar unit (Reeves et al. 2002a). The use of this HEK293S-GnTI⁻-TetR cell line, which is tetracycline-inducible and suspension adaptable, has become increasingly popular with regards to membrane protein production for structural studies purposes (Table III). This popularity is likely to continue considering that in the recent structure of a rhodopsin mutant, produced from material generated in the HEK293S-GnTI⁻ cell line, the $GlcNAc_2Man_1$ part of the sugar unit was not only ordered but also formed crystal contacts (Deupi et al. 2012), showing that well-diffracting crystals of membrane proteins may be obtained even if truncated N-glycans are present.

Conclusions and future perspectives

The tetracycline inducible system originally developed by Yao et al. (1998) has proven to be the most effective methodology to express mammalian membrane proteins in mammalian cells, both in terms of functionality and expression levels. This has resulted in structures of rhodopsin mutants (Standfuss et al. 2011, Deupi et al. 2012) and the human ammonia transporter (Gruswitz et al. 2010). This system has proved to be the most effective system for the production of even extremely complex membrane proteins like SERT that has a known dependence of cholesterol for activity and for N-glycosylation for efficient folding (Tate et al. 2003). It is likely that many more membrane proteins that are difficult to express in a homogeneous state in the baculovirus system will be expressed effectively using this tetracycline-inducible system.

The major issue of using tetracycline-inducible mammalian cells to produce membrane proteins is that expression levels are modest, requiring the routine growth of 10 l of cells, which is both costly and time consuming. In our lab, it costs about £1100 per 10 l of suspension-adapted HEK293 cells, compared to £600 for CHO or Sf9 cells (all prices using commercially prepared liquid media) and usually takes about two weeks per wave bioreactor. The development of TetR-expressing cell lines that grow routinely to greater than 10^7 cells/ml without perfusion or feeding regimes would be a great benefit, particularly for N-glycosylation deficient mutant cells. In this regard, there has been no systematic study of which cell lines are the most efficient for producing membrane proteins, although HEK293 cells are the most frequently used at the moment. However, it is clear that most membrane proteins have not evolved to be highly overexpressed as only a few thousand copies per cell may be sufficient for their biological role and to confer the

desired phenotype on the organism. Engineering membrane proteins to improve either their stability and/or expression (Magnani et al. 2008, Serrano-Vega et al. 2008, Shibata et al. 2009) is therefore a way forward to allow structural studies on virtually any membrane protein.

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