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Minireview

Overexpression of mammalian integral membrane proteins for structural studies

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Abstract Recent successes in the determination of atomic resolution structures of integral membrane proteins have relied on purifying the proteins from abundant natural sources. In contrast, the majority of mammalian receptors, ion channels and transporters need to be overexpressed to obtain sufficient material for structural studies. This has often proved to be very difficult. Overexpression studies on a wide range of mammalian membrane proteins have shown that a few can be expressed functionally in bacteria, but many others require an insect or mammalian cell host for activity or high level expression. The serotonin transporter, which has been expressed in all the major hosts available, is a good example that has given insights into the problem of overexpressing mammalian membrane proteins for structural studies.

Key words: Structure; Crystallization; Serotonin transporter

1. Introduction

The last 2 years have been exciting in the world of membrane protein structures with the structure determination at atomic resolution of three mammalian membrane proteins: the G protein-coupled receptor rhodopsin [1], the water channel aquaporin [2] and an ATP-dependent ion pump, the sarcoplasmic reticulum Ca^{2+}-ATPase [3]. One of the major factors in dictating why these particular membrane proteins were crystallised was their natural abundance, circumventing all the difficulties associated with overexpression. However, the majority of medically important membrane proteins are present in tissues at very low concentrations making overexpression a prerequisite for structural studies. Even for proteins like rhodopsin an overexpression system is desirable, because the structure of mutants will illuminate structural changes in the photocycle leading to G protein activation. The value of such structures in furthering ideas on the mechanism is clearly seen in the case of bacteriorhodopsin [4,5].

So what overexpression systems have been used to produce enough protein for crystallisation? An analysis of the mammalian membrane protein structures published shows that the majority of the proteins were purified from naturally abundant sources (Table 1). In contrast, many bacterial membrane proteins were overexpressed in related bacteria; note that halorhodopsin was overexpressed in *Halobacteria salinarum* (an archaebacterium) and not in *Escherichia coli* (an eubacterium). There is only a single example of an overexpressed mammalian membrane protein, gap junctions, leading to structural data [6], but in this instance the two-dimensional crystals formed in vivo in the mammalian expression system; the purification of gap junctions in milligram quantities from this cell line would have been much more difficult. This analysis agrees with the extensive survey of expression systems performed previously [7], which concluded that, in general, homologous expression is far better at producing *functional* membrane proteins than heterologous expression. I will discuss the relative merits of expressing non-functional protein and refolding at the end of the paper. To illustrate the problems with overexpressing mammalian membrane proteins, the serotonin transporter will be discussed.

2. The serotonin transporter

The serotonin transporter (SERT) is an example of a mammalian membrane protein that has been particularly difficult to overexpress. This section will give a brief introduction to SERT, followed by a discussion of some of the expression systems we have tried, hopefully giving hints and ideas that are applicable to the expression of other membrane proteins.

SERT is found in the presynaptic nerve termini where it terminates synaptic transmission by transporting serotonin back into the cell (reviewed in [8,9]). Uptake is driven by utilising the Na^+ and Cl^- gradients across the membrane. SERT contains 12 transmembrane domains, with the N- and C-termini intracellular. A large external loop between transmembrane regions 3 and 4 is *N*-glycosylated [10] and probably contains an intra-loop disulphide bond [11]. SERT is the site of action of antidepressant drugs, amphetamines and cocaine. The availability of tightly binding inhibitors has allowed accurate determination of *functional* expression levels, regardless of where in the cell the transporter is situated. Western blots have been used to determine total levels of SERT expression and the presence of *N*-glycosylation.

Expression of SERT was attempted in both *E. coli* and in the yeast *Pichia pastoris* [12]. The *E. coli* expression trials used two different constructs (GST-SERT and His_6-SERT), three different promoters (*tac*, *trc*, T7), three different temperatures (37°C, 30°C, 17°C) and five different *E. coli* strains. Although SERT could be clearly observed on Western blots of whole

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cell extracts at reasonable expression levels (~1 mg/l), only a small proportion of SERT was membrane associated. Binding assays using the inhibitor imipramine did not detect any Na^+-dependent high affinity binding indicative of correctly folded SERT; low affinity binding not observed in the absence of plasmid was found, but what this means in terms of SERT structure is unclear. Expression in *P. pastoris* gave similar results to expression in *E. coli*; clones in two different yeast strains (GS115, SMD1168) containing one to six copies of the SERT cDNA were produced, but no serotonin uptake was detected and only low affinity imipramine binding was found. Thus, in bacteria or yeast, functional expression of SERT was not observed.

The baculovirus expression system was used to express SERT in insect cells [10,13]. Functional SERT was detected by both uptake of ^{3}H-serotonin into intact cells and by ^{125}I-RTI55 inhibitor binding assays. There were about 250 000 functional molecules of SERT per cell when grown in shaker flasks. Changing external parameters such as medium composition, multiplicity of infection or cell type did not improve expression levels. Fusing the N-terminus of SERT to the C-terminus of a highly expressed protein, GST, actually led to a decrease in functional expression. The inclusion of apoptosis inhibitors did lead to slightly better cell survival, but expression levels were unchanged. Addition of SERT inhibitors to the cells also did not improve expression. The only improvement in expression levels obtained was a result of co-expression of the molecular chaperone calnexin, leading to a 3-fold increase in functional SERT [14,15].

One important clue to why SERT expression was problematic came from a Western blot of SERT expressed in four different insect cell lines (Sf9, Sf21, Hi5, MG1). The blot showed that there was nearly 100 times more SERT expressed in Hi5 and MG1 cells than in Sf21 cells, but the amount of functional SERT loaded per lane of the gel was identical [10]. The major difference between the samples was in the amount of unglycosylated SERT, suggesting that this was inactive. The implication was that *N*-glycosylation was important for the functional expression of SERT. This hypothesis was substantiated by removing the consensus *N*-glycosylation sequences in SERT by altering Asn to Gln [10]. Removal of both *N*-glycosylation sites led to a 20-fold decrease in functional SERT expression. However, other factors must also be important for SERT folding, because only about 10% of *N*-glycosylated SERT expressed in insect cells was functional, a conclusion drawn from comparative Western blotting and binding studies [14]. Despite the high level of misfolded SERT expressed, a purification based on Ni^{2+}-affinity chromatography produced only *N*-glycosylated SERT, substantiating the probability that the unglycosylated transporter was in an aggregated form in the cell (Tate, unpublished data).

Transient expression of SERT in mammalian cells, and the creation of stable cell lines, are the systems of choice for the analysis of mutants and the kinetic parameters for serotonin uptake and drug binding [16,17]. However, transient systems are difficult to scale up for the production of milligrams of protein, and the stable cell lines grow poorly and have to be maintained under stringent selection to ensure SERT expression. One possibility for the toxicity of SERT expression is that transport of serotonin found in the growth medium, or the transient channel-like activity described for SERT [18], results in significant stress for the cell, selecting against high-expressing clones. To test this hypothesis, stable cell lines were constructed using the EBNA-HEK293 expression system [19,20], but the cells were maintained in the presence of SERT inhibitors throughout the selection of clones (Tate, unpublished data). No clones were obtained in the absence of inhibitors. Clones grown in the presence of imipramine or cocaine expressed on average 250 000 copies per cell, but the cells grew poorly even in the presence of the inhibitors. These figures are probably an underestimate of the actual expression levels, because confocal microscopy showed that only about a quarter of the cells expressed high levels of SERT; all the SERT was seen at the plasma membrane, in contrast to the baculovirus expression system were all the SERT was in the endoplasmic reticulum (Tate, unpublished data). The presence of inhibitors has clearly facilitated the generation of stable cell lines, but the levels of expression were no higher than seen in the baculovirus expression system.

The most promising development in expression systems

Table 1
Some recently determined three-dimensional structures of integral membrane proteins[a]

Membrane protein (native source)	Resolution (Å)	Source for purification	Ref.
Aquaporin-1 (human)	3.8	Human red blood cells	[2]
Rhodopsin (cow)	2.8	Bovine rod outer segments	[1]
Calcium pump (rabbit)	2.6	Rabbit muscle	[3]
Glycerol channel, GlpF (*E. coli*)	2.2	OE[b] in *E. coli*	[41]
Halorhodopsin (*H. salinarum*)	1.8	OE in *H. salinarum*	[42]
Bacteriorhodopsin (*H. salinarum*)	2.0	*H. salinarum*	[43]
Mechanosensitive channel, MscL (*Mycobacterium tuberculosis*)	3.5	OE in *E. coli*	[44]
Potassium channel, KcsA (*Streptomyces lividans*)	3.2	OE in *E. coli*	[45]
Photosystem II (*Synechococcus elongatus*)	3.8	*S. elongatus*	[46]
Cytochrome *bc*1 complex (*Saccharomyces cerevisiae*)	2.3	*S. cerevisiae*	[40]
Cytochrome *c* oxidase (cow)	2.3	Bovine heart	[47]
F_1F_0-ATPase (*S. cerevisiae*)	3.9	*S. cerevisiae*	[48]
Photosynthetic reaction centre (*Rhodopseudomonas viridis*)	2.3	*R. viridis*	[49]
Fumarate reductase (*Wolinella succinogenes*)	2.2	*W. succinogenes*	[50]
Nicotinic acetylcholine receptor (*Torpedo marmorata*)	4.6	*T. marmorata*	[51]
Gap junctions (human)	7.5	OE in mammalian cells	[6]
Na^+-H^+ antiporter, NhaA (*E. coli*)	7.0	OE in *E. coli*	[52]
H^+-ATPase (*Neurospora crassa*)	8	*N. crassa*	[53]

[a]This is not an inclusive list; porin structures are not included and only a single example of many complexes are cited. See http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html for a detailed list.
[b]OE, overexpressed.

over the past few years has been the introduction of inducible expression in mammalian cells [21]. However, the disadvantage of these systems was either that the promoters needed to be very weak to ensure low basal expression in the uninduced state, or that the inducers were prohibitively expensive for large scale expression. One system that has overcome these problems is the cold-inducible system developed at Cytos [22]. A stable cell line is constructed using a temperature sensitive mutant replicase from the Sindbis virus and the desired cDNA downstream. At 37°C the viral replicase is inactive, but from 34°C to 29°C there is a linear increase in activity with decreasing temperature. We have used this system to create stable cell lines in suspension grown mammalian cells that express 250 000 copies per cell (Boorsma and Tate, unpublished data). Further analysis of the cell line is under way to improve the expression levels.

3. What parameters affect the expression of SERT in the various systems?

We have attempted to express SERT in all the major expression systems from bacteria to mammalian cells. The conclusion is that SERT cannot be functionally expressed in *E. coli* or *P. pastoris*, but SERT is active when expressed in insect or mammalian cells. One clear reason why expression in *E. coli* may be difficult is the importance of *N*-glycosylation in the folding of SERT (see above). However, non-glycosylated SERT is functional [10], so despite *E. coli* being unable to *N*-glycosylate proteins, it should have been feasible to express functional SERT. *P. pastoris* can *N*-glycosylate proteins, but no functional SERT could be detected, so clearly other factors must be important for the proper folding of SERT.

One important difference between mammalian cells, yeast and bacteria is their lipid composition. This is especially pronounced in the case of sterols: mammalian cells contain mainly cholesterol, yeasts contain mainly ergosterol and bacteria do not have any at all. The pertinence of sterols in SERT expression was suggested from reconstitution experiments on a related protein, the GABA transporter. Reconstitution in the presence of small amounts of cholesterol increased the initial rate of GABA uptake into the proteoliposomes by a factor of at least 20 [23]. The tiny amounts of cholesterol needed for this effect suggested that it was interacting specifically with the transporter, rather than the result being due to non-specific changes in the fluidity and thickness of the lipid bilayer. Is cholesterol essential for SERT activity? After removing cholesterol from insect cell membranes containing functional SERT, inhibitor binding activity was significantly reduced, but this could be partially restored by immediate addition of cholesterol [12]. However, addition of cholesterol analogues, such as ergosterol, did not restore binding activity. This suggests that there is indeed a specific requirement of cholesterol for SERT functionality. The absence of cholesterol in bacteria and yeast suggests a plausible reason for their inability to express functional SERT.

The level of functional SERT expression, whether it is in the baculovirus expression system, or as constitutive or inducible expression in mammalian cells, seems to be relatively independent of the system used and reaches a maximum of about 250 000 copies per cell. The only way found so far to increase expression levels higher than this was to co-express the molecular chaperone calnexin [14,15]. This suggests that the folding of SERT is the slowest step in the overexpression process. Functional expression of 250 000 copies per cell, therefore, may represent an equilibrium between the rate of expression and the rate of degradation. Co-expression of calnexin which increases the overall rate of folding in the cell would therefore increase expression levels. Another possibility is that 250 000 copies per cell represent the maximum load that the cell can tolerate in the plasma membrane without affecting the structural integrity of the cell. If this was true, then expressing SERT targeted to the endoplasmic reticulum might be expected to lead to higher expression levels.

4. How does SERT expression compare to other membrane proteins?

There seems to be a spectrum of 'express-ability' within membrane proteins ranging from the facile to the very difficult. This spectrum is probably derived from a variety of contributing factors including the difficulty of folding, rates of degradation and toxicity to the cell. It is not clear whether it will be possible to predict the ease of overexpressing a particular membrane protein just from its primary sequence. The complexity of a membrane protein might be thought to be related to the number of transmembrane domains; this is clearly untrue. Bacterial transporters of the major facilitator superfamily containing 12 transmembrane domains are easily overexpressed so that they represent 20–50% of the inner membrane protein of *E. coli* [24]. In comparison, members of the small multidrug resistance family contain only four transmembrane regions and yet they are expressed at less than one tenth the levels of the 12-helix transporters [25]. Another example, but this time in the baculovirus expression system, is the 20-fold difference in expression levels of the 7-helix muscarinic G protein-coupled receptors (GPCRs) [26], but all of these are expressed at lower levels than the 12-helix human glucose transporter GLUT1 [27]. Further examples are cited in Grisshammer and Tate [7].

The 'express-ability' spectrum should really be a log scale, because it is often orders of magnitude more difficult to express some membrane proteins compared to others. This is particularly true when considering the expression of mammalian membrane proteins in *E. coli* compared to expression of bacterial membrane proteins. The most studied examples of mammalian membrane proteins expressed in *E. coli* are GPCRs [28], whose functional expression levels can be accurately determined by ligand binding assays. The maximal level of GPCR functional expression is currently 3500 copies per cell, about 0.6 mg/l of cells [29], which is still over an order of magnitude lower than many bacterial membrane proteins. The reasons for this difference are unclear, but probably arise at the folding step, perhaps due to two effects. Firstly, the rate of polypeptide elongation in procaryotes [30,31] is 4–10 times faster than in eucaryotes [32,33], and this is parallelled by an increased rate of folding by procaryotic proteins [34]; synthesis of a mammalian polypeptide in *E. coli* would therefore be much faster than normal, resulting in more polypeptide present than usual at each stage of the folding process, which could give rise to misfolding. Secondly, the apparatus for inserting membrane proteins into the membrane, the translocon, is different in eucaryotes and procaryotes, despite some similarities [35,36]. It is not yet clear what specific interactions there are between the translocon and a nascent polypeptide

chain, and how these interactions may modulate the synthesis of an integral membrane protein. It is also unknown what effect subtle differences between eucaryotic and procaryotic membrane proteins have on heterologous expression; for example, the positive-inside rule is much less pronounced towards the C-terminus of membrane proteins in eucaryotes in comparison to procaryotes [37].

Inclusion bodies are an attractive way of producing large amounts of protein, but this naturally requires the refolding of the protein into a functional form before crystallisation, which has been achieved for only relatively few membrane proteins (reviewed in [7]). The expression level of membrane proteins forming inclusion bodies varies depending on the protein being expressed and factors affecting expression levels have been studied [38]. The refolding field was given hope by the apparent refolding of a mammalian GPCR, the odorant receptor OR5 [39]. Unfortunately the hydrophobic nature of the ligand and low affinity of binding made it difficult to do saturation binding assays for this receptor, so it was impossible to quantify the amount of refolded protein and to do a rigorous pharmacological analysis. Recently it has been claimed that other mammalian GPCRs have been refolded (www.mphasys.com), but the absence of published material makes it hard to critically evaluate the data. Clearly overexpression as inclusion bodies and refolding is an attractive way to produce large quantities of membrane proteins for structural studies, but it is likely that a similar spectrum of refolding ability will exist as it does for expression. It will be interesting to see if the refolding and expression spectra are identical, i.e. is the easiest membrane protein to refold also the most highly expressed in a functional form?

5. Conclusion

The recent successes in determining the structure of integral membrane proteins is due largely to recent advances in X-ray crystallography and, with the realisation that it is possible, a considerable increase in effort and resources is being expended towards other membrane proteins. However, the current state of overexpression technology means that bacterial membrane proteins are the most amenable for structure determination, simply because they can often be expressed easily in large quantities. In comparison, the overexpression of mammalian membrane proteins in bacteria is, at best, an order of magnitude lower, requiring large scale growth (tens of litres) to produce 1 or 2 mg of purified membrane protein. However, many mammalian membrane proteins cannot be expressed in *E. coli* or even in yeast. Currently these membrane proteins require the growth of tens of litres of insect or mammalian cells, which is extremely time consuming and costly compared to the growth of bacteria. It may be possible to engineer bacteria or yeast to functionally express these types of membrane, but it will require alterations to many complex systems involved in protein synthesis and folding. The refolding of membrane proteins is creating considerable interest at the moment; it remains to be seen how applicable this will be to the membrane proteins that are difficult to express in a functional form.

Overexpression is only the first problem encountered on the road from cDNA sequence to the structure of a membrane protein. The characteristics of a particular membrane protein not only seem to dictate expression levels, but they also have a profound effect on purification and crystallisation. It is perhaps no surprise that the membrane proteins crystallised so far tend to be rigid and stable for a long time in many detergents. The ability to rigidify supposedly flexible receptors and transporters, by using inhibitors or conformation-dependent antibody fragments [40], will no doubt play an important role in the future in obtaining further membrane protein structures.

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