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Review

Structural genomics for membrane proteins

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Abstract. Structure-based drug discovery has proven useful in improving and shortening the drug development process. The approach of structural genomics to study a large number of targets in parallel has been commonly applied to protein families and even whole genomes. Paradoxically, although membrane proteins represent the largest type of drug targets, up to 70% today, determination of their structure has been modest compared to that of soluble proteins. Because membrane proteins are important for drug discovery an emphasis has been placed on developing technologies and methods to determine membrane protein structures. Several structural genomics initiatives have been established, focusing on the structural biology of membrane proteins.

Keywords. Structural genomics, membrane proteins, networks, recombinant protein expression, purification, structure determination.

Introduction

Drug discovery is a long and risky process with many pitfalls. During the past 10 years, a variety of methods have been developed whose aims are to shorten the length of the process discovery and improve the efficacy and reduce the side effects of newly developed drugs. Classic drug discovery has consisted of selection of appropriate compounds, which are then subjected to biological evaluation in lead discovery [1]. Recently, a number of high-throughput *in vitro* screening methods and the synthesis of combinatorial compound libraries have further advanced the field. Structure-based drug design has played an important role in lead optimization [2], but high-resolution structures of the HIV proteinase [3] and influenza virus neuraminidase [4] have also directly contributed to such AIDS drugs as Agenerase and Viracept and the flu drug Relenza.

Although membrane proteins represent the most important drug targets, their structure determination has been very inefficient: among the more than 30,000 structures deposited in public databases today only some 100 represent membrane proteins [see http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html]. The reasons for the low success rate can be summarized as follows. Membrane proteins have generally been much more difficult to express as recombinant proteins than their soluble counterparts. While hundreds of milligrams of soluble protein could be produced in *Escherichia coli* or baculovirus-infected insect cells, the yields have been moderate for membrane proteins in almost all the expression systems tested [5]. More recently, although relatively high levels of membrane protein expression have been obtained in bacteria, yeast, insect and mammalian cells, this necessitated major engineering efforts [7]. Additionally, the transmembrane-integrated topology requires use of detergents during membrane protein purification, which has a negative impact on the yields and stability of the purified material. Furthermore, crystallization in the presence of detergents as well as the dynamic structure of many membrane proteins with flexible regions reduces the crystal contacts and thereby hampers the structure determination. Finally, although the methods applied in X-ray crystallography and nuclear magnetic resonance (NMR) have seen significant improvements, the available technology is still more suitable for soluble

proteins. Typically, only a single, high-resolution structure is available for the family of G protein-coupled receptors (GPCRs), even though they represent over 50% of current drug targets. Moreover, the only GPCR structure solved so far is the inactive form of bovine rhodopsin, present at high abundance in the retina, which allows direct purification from native tissue [7].

Further technological development has been required to improve the success rate with membrane protein structure determination. Major achievements have been obtained for certain targets. However, much time and effort has been required to engineer a multitude of gene constructs with various deletions, fusion partners and purifications as illustrated for the expression of the rat neurotensin receptor in *E. coli* membranes [8]. Another strategy commonly used today is to set up structural genomics networks, which due to their broad area of expertise and large handling capacity are able to study a large number of targets in parallel. The advantage of this procedure is that bioinformatics approaches may reveal why some membrane proteins are better expressed than others. Moreover, studies on a large number of targets in parallel should generate enough material for further purification and crystallization studies. The large networks often have the capacity to handle alternative expression systems in parallel, which further improves the success of producing structural biology-compatible material for further studies.

Structure-based drug design has not been possible to apply directly to targets for membrane proteins due to the lack of available three-dimensional (3D) structures. However, initial molecular modeling of several GPCRs was conducted on the structure of bacteriorhodopsin [9] and more recently based on the high-resolution structure of bovine rhodopsin [7]. Site-directed mutagenesis has given some information on the binding sites for a number of receptors. These approaches have provided some help in drug design, but evidently a 3D structure of a GPCR of therapeutic importance would substantially facilitate the development of more selective and efficient drugs.

Structural genomics networks

Both national and international networks bringing together experts in the fields of expression, purification and structure determination have been established (Table 1). In the USA several networks have been established that focus on whole genomes. For example, the Southeast Collaboratory on Structural Genomics (SECSG) studies the genome of *Caenorhabditis elegans* and the Center for Eukaryotic Structural Genomics (CESG) focuses on the genome of *Arabidopsis thaliana*. Many networks have selected a limited number of membrane proteins in addition to their mainstream activities on the structural biology of soluble proteins. In these attempts, a limited effort is

Table 1. Selected examples of structural genomics networks with a specific interest in membrane proteins.

Network	Expression system(s)	Membrane protein targets
E-MeP www.e-mep.org	*E. coli*; *Lactococcus lactis*; *Saccharomyces cerevisiae*; *Pichia pastoris*; baculovirus; Semliki Forest virus (SFV)	100 prokaryotic MPs 100 GPCRs 100 non-GPCR eukaryotic MPs
MePNet Membrane Protein Network www.mepnet.org	*E. coli*; *P. pastoris*; SFV	100 GPCRs
JCSG Joint Center for Structural Genomics www.jcsg.org	cell free; *E. coli*; adenovirus; baculovirus; SFV	*Thermotoga maritima* proteome; mouse genome; human GPCRs
Membrane Protein Platform www.swegene.org	*E. coli*; *S. cerevisiae*; *P. pastoris*	bacterial, yeast and plant MPs; human GPCRs
CESG Center for Eukaryotic Structural Genomics www.uwstructuralgenomics.org	*E. coli*	*Arabidopsis thaliana* genome
SECSG Southeast Collaboratory on Structural Genomics www.secsg.org	*E. coli*; baculovirus; lentivirus	*Pyrococcus furiosus* genome *Caenorhabditis elegans* genome
NCCR Swiss National Center of Competence in Research www.structuralbiology.ethz.ch	*E. coli*; baculovirus	bacterial MPs; transporters; GPCRs
RIKEN Structural Genomics www.rsgi.go.jp/rsgi_e/index.html	*E. coli*; cell free; baculovirus	*Thermus thermophilus* genome mammalian GPCRs

Additional networks for membrane proteins exist, but these are the key consortia. MP, membrane protein.

dedicated to expression evaluation, which generally relates solely to the application of yeast or insect cell-based expression systems.

There are some networks which focus uniquely on membrane proteins. In this context, the EU-funded E-MeP program has 18 member teams and has selected 100 prokaryotic and 200 eukaryotic membrane proteins as their targets [10]. Prokaryotic membrane proteins are generally relatively well expressed in bacteria and have therefore provided higher success rates in structure determination than their eukaryotic counterparts. Several E-MeP members also have plenty of previous experience with prokaryotic membrane proteins such as the L-fucose-proton symport protein FucP [11]. Furthermore, the experience acquired from studies on prokaryotic membrane proteins might be applicable to their eukaryotic orthologs. In E-MeP, 100 prokayotic membrane proteins from various bacterial species are studied. The 200 eukaryotic targets consist of 100 GPCRs and 100 non-GPCRs representing ion channels, transporters and other types of transmembrane protein. The initial expression of selected eukaryotic targets is carried out as a comparative study in six different expression systems including bacterial expression in *E. coli* and *Lactococcus lactis* and yeast-based expression in *Saccharomyces cerevisiae* and *Pichia pastoris*. Insect cells are infected by baculovirus and mammalian cell lines by Semliki Forest virus (SFV). A cell-free translation system will be evaluated for just a few targets. Already existing 'pipelines' for purification and crystallization within the network will be filled quickly and targets processed efficiently. A successful example from the E-MeP network is the recently solved structure of the spinach aquaporin SoP1P2 channel expressed in *P. pastoris* [12].

Another network, uniquely focused on eukaryotic membrane proteins, is the privately funded MePNet (Membrane Protein Network) [13]. All 100 targets in MePNet are GPCRs and they have been evaluated for expression in three well-established expression systems. The expression in *E. coli* is directed to inclusion bodies, whereas the *P. pastoris*- and SFV-based expression is aimed at yeast and mammalian cell membranes, respectively. A comparative study indicated that the success rate of expression in *E. coli* was much lower (approximately 50%) than for *P. pastoris* (94%) and SFV (95%) [14]. Optimized expression of 20 GPCRs in *P. pastoris* from the MePNet program showed high binding activity (up to 76 pmol/mg) and has been described in detail elsewhere [15]. Furthermore, evaluation of 101 GPCRs expressed in SFV followed by binding studies on 42 GPCRs generated high yields of a large number of receptors that were functionally active [16]. Overall, the MePNet program produced some 60 GPCRs at levels considered compatible for structural biology studies.

Expression

For a long time, of the most serious bottlenecks in the structural biology of membrane proteins has been the recombinant production of membrane proteins to provide a sufficient quantity and quality of material. Due to the low abundance in native tissue, with a few exceptions such as bovine rhodopsin [7] and the nicotinic acetylcholine receptor from the electric organ of *Torpedo marmorata* [17], it has been necessary to produce the protein in heterelogous expression systems. To exemplify the problem: it was estimated that to purify 200 μg of the porcine neuropeptide Y2 receptor, 1000 pig brains were needed [18].

The problems with the expression of heterologous membrane proteins have been related to their toxic effect on host cells especially when inserted in bacterial membranes. The toxicity and other factors have contributed to low yields and poor stability of the recombinant membrane proteins. One approach to solve this issue has been to generate various fusion constructs and to introduce deletions and mutations in the recombinant proteins as a means of improving yields and stability [8]. Alternatively, a variety of non-bacterial expression systems have also been verified as expression hosts. The choice of expression system must also be assessed in relation to the post-translational modifications characteristic of mammalian membrane proteins. For example, the lack of many post-translational modification functions in prokaryotes can significantly affect the activity of the protein. On the other hand, it is well documented that glycosylation generates heterogenous recombinant protein and may hamper the crystallization efficacy of the purified protein. Additionally, lower eukaryotes such as yeast have different glycosylation patterns to mammalian cells, often resulting in a tendency to hyperglycosylate. For this reason, prokarotic expression of non-glycosylated protein might be advantageous. Alternatively, engineering of non-glycosylated mutants or deglycosylation of recombinant protein can be carried out in eukaryotic cells. The expression systems used for membrane protein production and the characteristics of the membrane proteins produced are summarized below and in Tables 2 and 3.

Bacterial expression

Recombinant protein expression has been most frequently carried out in *E. coli* due to the easy procedure, simple and cheap scale-up and high safety standards [19]. In the case of membrane protein expression, two approaches have been taken. The expression has either been targeted to the bacterial membrane or to inclusion bodies. For prokaryotic membrane proteins, the yields in membranes have been relatively good and a high degree of functionality

Table 2. Seleceted examples of expression systems applied for membrane proteins.

Host	Vector/promoter	Advantages	Disadvantages
Cell-free			
E. coli S30	T7	rapid, controlled	expensive scale-up
Prokaryotic			
E. coli	T7, ptac, lac Gateway/T7	easy, rapid, cheap can be scaled up	lack of post-translational modifications
Halobacterium salinarum	bop	rapid, colored product	*fusion proteins required*
L. lactis	nisA	Gram+ single membrane	*lack of post-translational modifications*
Eukaryotic			
Yeast			
S. cerevisiae	ADH, GAL1	can be scaled up	hyperglycosylation
Schizosaccharomyces pombe	DDH, NMT1	inexpensive	thick cell wall
P. pastoris	AOX1	inducible	clone selection
Insect			
Sf9, S21	baculovirus	mammalian-like post-translation	slow, expensive production
Schneider cells	methallothionin	GFP fusion	time consuming
Drosophila	transgenic	location in retina	time consuming
Amphibian			
Xenopus	transgenic	location in retina	time consuming
Mammalian cells			
Transient	CMV	mammalian cells	expensive
Stable cells	CMV, T-Rex	inducible	time-consuming
Viral			
Adenovirus	Ad	broad host range	slow virus production
Lentivirus	TExT™	broad host range	slow virus production
SFV	SFV26S	rapid, broad host range	expensive virus production
Vaccinia	T7, vaccinia	broad host range	safety concerns

bop, bacterio-opsin promoter; TExT™, TranzExpression Technology; GFP, green fluorescent protein; CMV, cytomegalovirus.

obtained [20]. In contrast, eukaryotic membrane proteins when inserted in bacterial membranes have often resulted in toxic effects on host cells, significantly reducing the yields. Extensive and time-consuming engineering of fusion proteins, deletions and purification tags has improved the yields substantially. For example, when the N-terminally deleted rat neurotensin receptor was fused to the maltose-binding protein (MBP), milligram quantities of receptor could be produced in *E. coli* [21]. Likewise, a C-terminally truncated adenosine A2a receptor fused to MBP generated 10–20 nmol/l receptor in bacterial inner membranes [22]. The alternative approach to express the recombinant protein in bacterial inclusion bodies has often resulted in high yields, but requires a refolding procedure to restore the functional activity of the aggregated protein [23]. Refolding of membrane proteins is difficult and inefficient although with recent technology improvement, promising results have been obtained for several GPCRs [24–26].

In addition to *E. coli*, other bacteria have also been tested for membrane protein expression. *Halobacterium salinarum*, the producer of bacterio-opsin protein (Bop), was used to overexpress *E. coli* aspartate transcarbamylase (AT) as a Bop-AT fusion protein at yields of 7 mg/l. However, when the yeast Ste2 receptor and human GPCRs were expressed from similar constructs, the yields were disappointingly low [27]. Another potentially interesting prokaryotic host is the Gram-positive bacterium *L. lactis*. Vectors based on the *L. lactis* NisA promoter and NisR and NisK regulatory trans-acting factors have demonstrated high levels of expression of ABC transporters and major facilitator subfamily efflux pumps [28]. Yeast mitochondrial transporter proteins have also been expressed in a functional form in *L. lactis* [29].

Expression in yeast cells

Different yeast strains have been frequently used for recombinant protein expression particularly because yeast culturing yeast and the production of large biomasses are comparable to those of bacteria. Moreover, yeast is a eukaryotic organism that possesses many essential post-translational mechanisms required for functionality of

Table 3. Expression of membrane proteins: selected examples of GPCRs expressed in different systems.

Membrane protein	Yield	Expression system	Reference
Adrenergic β2	6 pmol/mg	*E. coli* membranes, MBP fusion	96
β2	115 pmol/mg	*S. cerevisiae*	97
α2C	7–70 pmol/mg	*S. cerevisiae*	32
β2	25 pmol	*P. pastoris*	98
β2	40 pmol/mg	baculovirus/Sf9 cells	99
β2	200 pmol/mg	stable CHO cells	54
α2B	176 pmol/mg; 10 mg/l	SFV/CHO cells	100
Adenosine A2a	10–20 nmol/l	*E. coli* membranes, MBP fusion	22
A2a	180 pmol/mg	*P. pastoris*	15
A2a	270 pmol/mg	baculovirus/High Five cells	44
A2a	287 pmol/mg; 1–5 mg/l	SFV/BHK cells	16
Dopamine D2	14 pmol/mg	*S. pombe*	35
D1	33 pmol/mg	baculovirus/Sf9 cells	101
D3	20 pmol/mg	SFV/CHO cells	102
Glutamate R2, R4	15–30 pmol/mg	baculovirus/Sf9 cells	103
Histamine H1	85 pmol/mg	baculovirus/Sf21 cells	44
H2	80 pmol/mg	SFV/COS7 cells	104
Neurokinin	80 pmol/mg; 1 mg/l	baculovirus/Sf9 cells	43
	100 pmol/mg; 10 mg/l	SFV/CHO cells	63
Neuropeptide Y	1 mg/l	vaccinia/HeLa cells	105
Neurotensin	15 pmol/mg	*E. coli* membranes, MBP fusion	21
Rhodopsin	4 mg/l	baculovirus/Sf9 cells	42
	10 mg/l	stable HEK293S cells	53
Opioid μ	100 pmol/mg	*P. pastoris*	106
Serotonin 5HT5A	40 pmol/mg	*P. pastoris*	107
5HT1A	5–34 pmol/mg	baculovirus/Sf9 cells	108
5HT1E	15–40 pmol/mg	HEK293 cells	109
TRH receptor	2×10^6 receptors/cell	adenovirus	110
TSH receptor	2.5 mg/l	*E. coli* membranes, fusion	111

TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone, MBP, maltose-binding protein.

many mammalian proteins. *S. cerevisae*, or Baker's yeast, is well characterized and has been used for a large number of membrane proteins. The yeast Ste2 receptor [30] and the human dopamine D1A receptor [31] have been expressed at high levels in *S. cerevisiae* and the receptors could be purified to high homogeneity. Likewise, the α2 adrenergic receptor was produced in 15-l fermentor cultures at yields of 20–30 mg of functionally active receptor [32]. Recently, the rabbit sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase 1a was expressed in *S. cerevisiae* and further purified and crystallized to give a structure determination at 3.3-Å resolution [33].

Because of plasmid instability and hyperglycosylation of recombinant proteins in *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* has been used as an alternative host [34]. Comparative studies on the human dopamine D2 receptor in *S. cerevisiae* and *S. pombe* revealed fivefold higher binding activity in fission yeast [35]. Although the B_{max} value was 14.6 pmol/mg in this case, expression of the rat dopamine D2 receptor [36] and the human neurokinin-2 receptor [37] resulted in levels of only 1 pmol/mg in *S. pombe*.

Definitely the biggest impact from yeast hosts is provided by *P. pastoris,* with more than 200 recombinant proteins expressed in this methylotrophic yeast strain [38]. A number of GPCRs have been expressed at levels of 20–40 pmol/mg in *P. pastoris* [39]. Expression optimization of 20 of the 100 GPCRs expressed within the MePNet consortium resulted in up to 76 pmol/mg receptor expressed from *P. pastoris* vectors [15]. Recently, structures of several membrane proteins expressed in *P. pastoris* have been successfully solved. For example, the spinach aquaporin SoP1P2 structure was solved at 3.3Å [12] and a mammalian voltage-dependent K^+ channel from the Shaker family at 2.9-Å [40] resolution.

Insect cell expression

Insect cells have frequently been used as expression hosts because of their close resemblance to mammalian cells. Baculovirus vectors, in particular, have been applied because of the relatively easy recombinant virus production and scale-up process of insect cells [41]. Several GPCRs

have been expressed from baculovirus vectors. For example, rhodopsin was expressed at yields of 4–6 mg/l, 80% of which was in a functional form [42]. Binding studies on the neurokinin-1 receptor revealed up to 60 pmol/mg receptor [43]. A study of 16 human GPCRs expressed in three (Sf9, Sf21 and High Five) insect cell lines infected with baculovirus vectors demonstrated variation in binding activity from 1 to 250 pmol/mg [44].

In addition to baculovirus vectors, stable *Drosophila* Schneider cell lines have been applied for recombinant protein production [45]. The N-terminally green-fluorescence protein-tagged human mu opioid receptor showed the same pharmacological profile in Schneider 2 cells as in mammalian cells, and the functional coupling to G proteins was demonstrated by cAMP stimulation and [^{35}S]-GTPγS-binding experiments [46]. Based on quantitative fluorescence intensity analysis, a large amount of the receptor was apparently retained in intracellular structures.

Non-viral and viral vectors for mammalian cells

Expression in mammalian cells is obviously as close as one can get to the native environment for mammalian proteins. The drawbacks of using mammalian cell lines have, however, been the low yields, high costs and time-consuming procedures. Both transient and stable expression has been established in mammalian cell lines. A number of GPCRs, such as the cholecystokinin A receptor, have been expressed in COS [47] and HEK293 cells [48]. The expression levels have been in the range of 1 to 10 pmol/mg. Other types of membrane protein have been expressed transiently. For example, the GABA transporter GAT-1 was expressed in Ltk cells [49], the muI Na^+ channel in HEK293 cells [50] and GLUT transporters in COS-7 cells [51]. Although large-scale production has been considered as a bottleneck for transient mammalian systems, recent improvements have made it more feasible [52].

The convenience of using stable cell lines has been hampered by the time-consuming process of generating them and the modest expression levels. Nevertheless, quite a few membrane proteins have been expressed at relatively high levels, although the total yields for GPCRs have been rather low (in the range of 0.1 mg/l). However, a mutant HEK293 cell line has generated up to 6 mg/l of rhodopsin [53]. Moreover, the human β2 adrenergic receptor was expressed at very high levels in a stable CHO cell line [54]. B_{max} values of close to 200 pmol/mg were observed, indicating that the recombinant receptor represented more than 1% of the total membrane protein. Verification of the serotonin transporter (SERT) protein has been carried out in various stable expression systems. In this context, the Sindbis virus-based, cold-inducible pCytTS system generated 250,000 copies/cell of SERT and the tetracycline-inducible T-Rex system produced 400,000 copies/cell [55].

Viral vectors have been attractive for recombinant expression due to their broad host range and high expression levels. Membrane proteins have frequently been expressed from adenovirus, vaccinia virus, lentivirus and alphavirus vectors in different types of mammalian cell lines and primary cells [56]. When the mu and kappa opioid receptors were expressed from adenovirus vectors in CHO cells, the pharmacological properties were identical to those of receptors from native tissue, but the binding values were threefold higher (3 pmol/mg) than in stable cell lines [57]. The α2 adrenergic receptor has been expressed at 4 pmol/mg in rabbit myocytes [58]. Vaccinia virus has been applied for the expression of several GPCRs. For example, expression of the neuropeptide Y receptor generated 5–10 million binding sites per cell, and the receptors were localized at the plasma membrane [59]. Moreover, functional activity was described for the human dopamine D2 and D4 receptors after vaccinia virus infection of rat-1 cells [60]. Lentivirus vectors have shown particularly efficient long-term expression in cell lines and *in vivo* [61]. The human retinal pigment epithelium GPCR has been expressed from a replication-deficient lentivirus vector [62]. The expression in ARPE-19 (retinal pigment epithelium) was 100-fold higher than in COS-7 cells, and stable expression was observed for up to 6 months. Recently, several GPCRs have been expressed successfully using the lentivirus-based TranzExpression Technology (TExT^{TM}) [www.tranzyme.com]. Probably the most used viral system for membrane protein expression is based on the replication-deficient alphavirus named Semliki Forest virus (SFV). The rapid, high-titre virus production and broad host range are particularly interesting features of SFV. A large number of GPCRs and ion channels have been expressed from SFV vectors, with yields up to 10 mg/l of recombinant membrane protein in bioreactor and spinner flask cultures [63]. Within the MePNet program, 101 GPCRs have been evaluated for expression [16]. The highest binding value of 287 pmol/mg was obtained for the human adenosine A2a receptor, and many GPCRs could be produced at levels of 1–10 mg/l for further structural biology applications.

Cell-free translation

Although cell-free translation systems have presented a serious alternative for the structural biology of soluble proteins, especially in the area of isotope labeling for NMR [64], the expression levels have been insufficient for membrane proteins. Recent modifications of *E. coli* S30 extracts have, however, significantly improved membrane protein yields [65]. The bacterial multidrug trans-

porter proteins TehA and YfiK were expressed at yields of 2.7 mg/ml. Several GPCRs, the human β2 adrenergic receptor (β2AR), the human muscarinic acetylcholine M2 receptor (M2R) and the rat neurotensin receptor (NTR) have been evaluated in the cell-free system [66]. Functional binding activity was obtained only after fusion of the α2AR to Gαs [67], the M2R to Gαi [68] and the NTR to MBP [21].

Alternative expression systems

In addition to the established expression systems described above, more exotic approaches have been taken. In this context, GPCRs have been successfully overexpressed in fruit fly eyes [69]. This has been achieved by generating transgenic *Drosophila melanogaster* expressing heterologous GPCRs in the photoreceptor cells. For example, the *D. melanogaster* metabotropic glutamate receptor (DmGluRA) was successfully expressed in photoreceptor cells. DmGluRA could be purified in a functional form from fly eyes at high yields. The yields were threefold higher than from baculovirus vectors in insect cells.

Another approach has been to overexpress GPCRs in frog eyes. Twenty GPCRs were expressed in the retina rod cells of transgenic *Xenopus laevis* [70]. The heterologous GPCRs localized to the rod outer segments and were homogenously glycosylated. The 5-HT1A and EDG1 receptors showed specific binding activity and coupling to G protein in [^{35}S]-GTPγS binding assays. As all transgenic *X. laevis* constructs with exogenous GPCRs contained a rhodopsin-specific C-terminal immunoaffinity tag, the receptors could be purified to high homogeneity. Furthermore, an automated system for generating hundreds of transgenic tadpoles per day was developed.

Purification

Another complicated area for membrane proteins is purification, because solubilization is required to separate the protein of interest from lipid components [71]. Detergents are used for this procedure and the general experience is that each protein has a specific preference for detergents or a detergent mixture, which therefore requires extensive screening processes [72, 73]. After solubilization the recombinant protein is commonly purified by affinity chromatography methods. Engineering of histidine-tags at the N or C terminal of the protein of interest allows the application of immobilized metal affinity chromatography [74]. Alternatively, streptavidin, biotin, FLAG and hemagglutinin tags have been used [73]. Where antibodies or ligands to receptors are available, affinity columns based on these molecules can also be used.

In addition to affinity tags, classical biochemical purification methods such as ammonium sulfate precipitation and sucrose gradients can be applied. The drawback is that these methods require large quantities of protein. Furthermore, ion exchange chromatography, gel filtration or size exclusion chromatography, hydrophobic interaction methods and reverse-flow chromatography are possible alternatives for protein purification.

Structure determination

X-ray crystallography is the method of structure determination reaching the highest resolution (<2.0 Å). Crystallography has various steps, including crystallization of highly purified protein, measurement of crystal diffraction, solving phase determination problems, phase and electron density calculations and model building and refinement. Automation and miniaturization have had a major impact on the crystallization process, allowing numerous crystallization conditions to be screened in parallel and reducing the amount of purified protein required [75]. This development now allows 100,000 crystallization trials to be conducted per day. Important issues that crystallographers still have to deal with are the harvesting and storage of crystals, data collection at beam lines and crystal characterization. The miniaturization process will probably also lead to the production of smaller crystals for which microdiffractometer technologies have to be developed.

Detergents play a critical role in the success of crystallization, because membrane proteins have to be extracted from their lipid bilayer. Moreover, detergents can self-assemble to form micelles, bicelles, lipid bilayers or lipid vesicles [76]. Through the protein-detergent complex formation, the crystallization of membrane proteins can be facilitated [77]. However, micelles require space in the crystal lattice, which might reduce the potential crystal contacts. To enlarge the polar surfaces of membrane proteins, domains of specifically binding antibody fragments have been engineered [78]. It has been clearly demonstrated that detergent and lipid selection are of the outmost importance. For this reason, much emphasis has been put on the development of new detergents, and lipopeptides [79], amphipols [80] and tripod amphiphiles [81] may prove useful. In attempts to reproduce the native environment of the protein of interest in the lipid bilayer, lipidic cubic-phase technology based on gel-like materials has been employed [82]. Three-dimensional bilayer structures are arranged to form topologically distinct lipid and aqueous regions. Addition of protein and precipitating agents induce crystallization. Archeal 7TM helix proteins [83] and light-harvesting complexes and photosynthetic reaction centers [84] have been successfully crystallized using lipidic cubic-phase methods.

NMR has been a complementary technology to X-ray crystallography [85]. Despite poorer resolution and molecular-weight limits, NMR has routinely been used in lead compound identification and evaluation [86]. Larger proteins have recently become feasible for NMR studies through developments in probe and software technologies. NMR technology itself has made it possible to apply NMR for generation of iterative protein-ligand complexes [87]. Furthermore, the use of NMR for membrane proteins has entered a new era through novel solid-state and solution NMR methods [76].

Finally, atomic force microscopy (AFM) and electron microscopy have been applied in structural biology [88]. Bacteriorhodopsin [89] and the aquaporin AQP1 [90] have been reconstituted in two-dimensional crystals applying cryoelectron microscopy at 3.5-Å resolution, their structure being later confirmed by X-ray crystallography. AFM has also been applied to native and reconstituted membranes in aqueous solutions to study disc membranes of vertebrate photoreceptor rod outer segments [91]. These studies revealed that rhodopsin was present as dimers and higher oligomeric forms. Electron microscope tomography has been commonly used to produce 3D reconstructions of organelles [92]. More recently, the technology has found applications for molecules, especially supramolecular assemblies [93]. As an example of applying electron microscopy and single-particle reconstruction, the rat glutamate transporter GLT-1 was expressed in BHK cells from SFV vectors [94]. This study resulted in a low-resolution structure of GLT-1, and the 3D reconstruction was compared with the structure available for the transporter homolog GltP from *Pyrococcus horikoshii*. The comparison indicated that although the volumes of both GLT-1 and GltP were of similar dimensions, there was an additional density observed for the GLT-1 volume.

Conclusions and future aspects

Structure determination of membrane proteins has been relatively unsuccessful compared to that of soluble proteins. Among the more than 30,000 structures available today, only some 100 are for transmembrane proteins. The majority of these high-resolution structures are for bacterial proteins and very few structures are eukaryotic targets, which are obviously of significant interest as drug targets. To solve the discrepancy between available structure determination technologies and the therapeutic demand for protein structures to apply structure-based drug design, much effort has been dedicated to solving the bottlenecks in the three major areas – expression, purification and structure determination. Despite many excellent individual efforts in the field, a popular and seemingly fruitful trend has been to apply the approach of structural genomics to tackle issues related to membrane proteins. In a recent review, 11 structural genomics networks, not specifically dedicated to membrane proteins, were evaluated and it was clearly suggested that the approach was successful [95]. High hopes are therefore also placed on structural genomics for membrane proteins.

What then can we expect from the structural genomics efforts on membrane proteins in the future? A parallel can be drawn with the early days of structure determination of soluble proteins. In the 1970s, progress was slow, with only 5 high-resolution structures available in 1973, and 60 structures 6 years later. Only with major technology development was the growth in the number of structures almost exponential, from a few thousand in the middle of the 1990s to over 30,000 in 2005. Will we experience the same scenario for membrane proteins? Major technology development is underway, and structural genomics initiatives will no doubt make a substantial contribution to it. However, the current concern is that although we can today produce hundreds of milligrams of recombinant membrane proteins in various systems and several membrane proteins have been purified to high homogeneity, structure determination is still very difficult. There are still issues to be solved such as lipid composition and other factors related to the quality and stability of the purified membrane proteins. A common approach is to develop a technology for membrane proteins, which involves cocrystallization with novel antibodies, toxins, ankyrins and anticalins specific for membrane proteins. Time will tell whether, in the near future, we will be able to conquer the last frontier in structural biology and use this knowledge for the development of better medicines.

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