

Review

Semliki Forest virus vectors for rapid and high-level expression of integral membrane proteins

Kenneth Lundstrom

Bio-Xtal, Chemin de Croisettes 22, CH-1066 Epalinges, Switzerland

Received 26 July 2002; accepted 4 November 2002

Abstract

Semliki Forest virus (SFV) vectors have been applied for the expression of recombinant integral membrane proteins in a wide range of mammalian host cells. More than 50 G protein-coupled receptors (GPCRs), several ion channels and other types of transmembrane or membrane-associated proteins have been expressed at high levels. The establishment of large-scale SFV technology has facilitated the production of large quantities of recombinant receptors, which have then been subjected to drug screening programs and structure–function studies on purified receptors. The recent Membrane Protein Network (MePNet) structural genomics initiative, where 100 GPCRs are overexpressed from SFV vectors, will further provide new methods and technologies for expression, solubilization, purification and crystallization of GPCRs.

© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Semliki Forest virus; Heterologous gene expression; Integral membrane protein; Large-scale production; Purification; Structural biology

1. Introduction

The completion of the draft sequence of the human genome has generated vast amounts of sequence information, which awaits further analysis to enhance the understanding of the function of individual genes and proteins, and their interactions. Although sophisticated bioinformatics programs have been developed, it is essential to study the function of these sequences *in vivo* by recombinant protein overexpression approaches. The development of efficient expression systems has, therefore, been essential.

During the past 10 years, a multitude of expression vectors and host cell systems have been engineered, spanning from simple prokaryotic vectors to complicated eukaryotic expression methods. Obviously, bacterial expression systems, especially those based on *Escherichia coli* vectors, have been used frequently. However, these have turned out to be less suitable for overexpression of mammalian transmembrane proteins. The main reason for this is the many post-translational modifications and processing events required for correct folding of mammalian proteins, which

prokaryotic cells lack. Additionally, insertion of recombinantly expressed mammalian membrane proteins into bacterial membranes has caused severe toxicity to the host cells. Alternatively, overexpressed transmembrane proteins can be guided to form inclusion bodies in *E. coli*. The drawback of this approach is that extensive and difficult refolding exercises are required to restore the function of the recombinant receptors. Archaeobacteria, such as *Halobacterium salinarum*, have been developed as expression vectors for heterologous proteins [1]. Because *H. salinarum* naturally expresses large quantities of bacteriorhodopsin, it was thought that also mammalian transmembrane proteins could be well expressed in this organism. Disappointingly, even fusions between bacteriorhodopsin and mammalian transmembrane receptors resulted in only modest expression levels [2]. Other types of expression systems that have received increasing attention are those based on yeast cells. *Saccharomyces cerevisiae* has proven useful for expression of some transmembrane receptors although the glycosylation pattern is quite different from the one seen in mammalian cells [3]. Application of *Schizosaccharomyces pombe* as a host has brought vectors with a higher resemblance to mammalian glycosylation modes [4]. Moreover, expression of several G protein-coupled receptors (GPCRs) from *Pichia pastoris* has been highly successful [5]. Other

E-mail address: kenneth.lundstrom@mepnet.org (K. Lundstrom).

expression systems frequently used for transmembrane proteins are based on Baculovirus vectors in insect cells [6].

Many attempts have also been carried out to develop mammalian expression systems, which possess the capacity of proper folding and processing of mammalian proteins. However, many of the transient and stable expression systems engineered have not lived up to the expectations. Typically, conventional stable cell lines are time-consuming to establish and the expression levels are modest. Furthermore, stability problems occur frequently, because the gene expression cassette integrated into the host cell genome is inactivated or deleted. Although the expression levels are higher for transient systems, the gene delivery efficacy varies from one cell line to another and especially large-scale applications requiring suspension cultures are inefficient. In contrast, viral vectors possess some attractive features such as the high efficiency of gene delivery to many different mammalian host cell lines and the use of generally very strong promoters responsible for high transgene expression levels. Naturally, the drawback with viral vectors is their potential biosafety risks.

This review focuses on the use of Semliki Forest virus (SFV) vectors although similar expression systems have been engineered for other alphaviruses such as Sindbis virus [7] and Venezuelan Equine Encephalitis virus [8]. The major

issue described here is the application of SFV vectors for overexpression of transmembrane proteins. Various examples are given of applications for structural and functional studies based on the production of large quantities of recombinant receptors in mammalian suspension cell cultures. Furthermore, SFV vectors can be used for several other applications not described in this review, such as for various neurobiological studies, vaccine production and gene therapy applications which are reviewed elsewhere [9,10].

2. SFV expression vectors

SFV is a single-stranded RNA virus with an envelope structure. The SFV genome has been introduced into two plasmid vectors as cDNA copies [11] (Fig. 1). The expression vector contains the SFV nonstructural genes (nsP1–4), the strong subgenomic SFV 26S promoter and a multilinker cloning region for the introduction of foreign genes of interest. The SFV structural proteins are provided from the helper vector containing the capsid and envelope protein genes. Co-transfection of in vitro transcribed recombinant and helper RNA into BHK cells generates high-titer (10^9 – 10^{10} infectious particles/ml) recombinant SFV particles. These particles are capable of infecting a broad range of

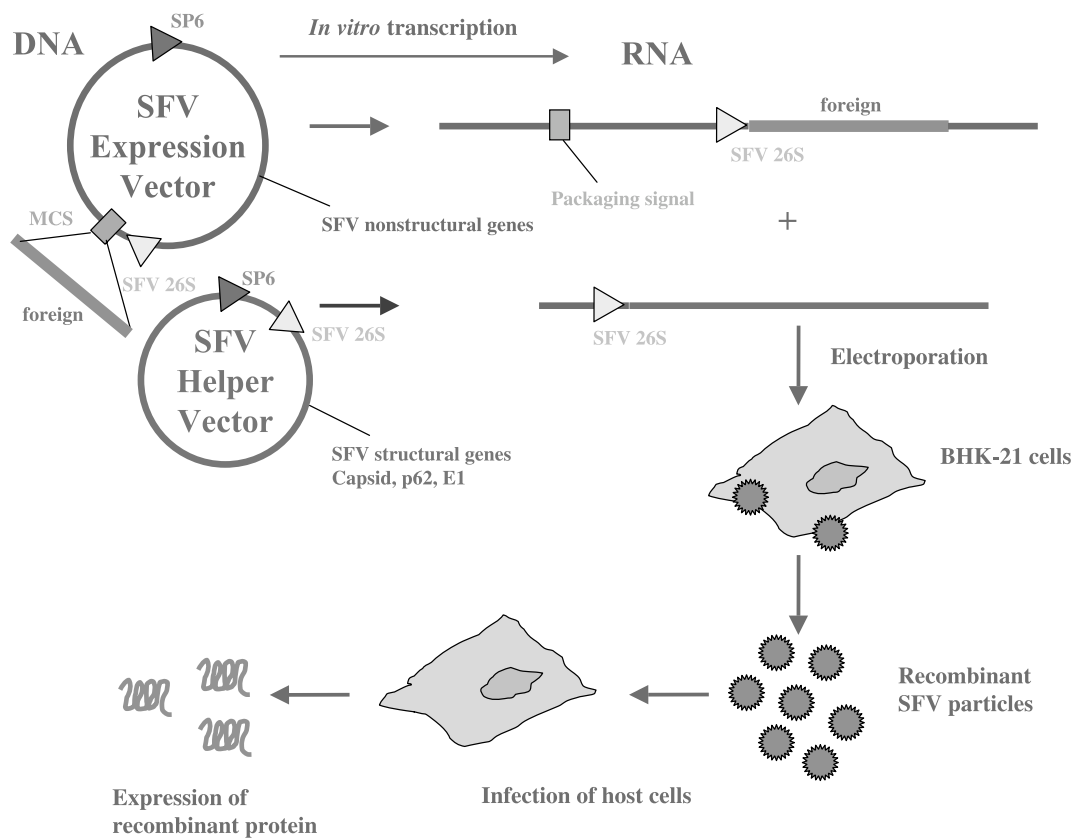


Fig. 1. Schematic illustration of the production of replication-deficient SFV particles. Recombinant and helper RNAs are in vitro transcribed from corresponding linearized DNA plasmids. BHK cells are co-electroporated and recombinant SFV particles harvested. Generated particles can be used for infection of various cell lines and primary cell cultures to produce high-level recombinant protein expression.

mammalian and other cell lines (amphibian, reptilian, insect, avian and fish) as well as primary cells. However, due to the presence of the RNA packaging signal only in the recombinant RNA, no helper RNA will be packaged, which renders the generated particles replication deficient. Upon infection of host cells, rapid and high-level transgene expression is obtained from SFV vectors without the generation of virus progeny.

Typically, recombinant SFV particles can infect many mammalian host cell lines generally used for the expression of recombinant proteins [12]. One advantage of using SFV is the possibility to perform expression studies in several cell lines in parallel. Moreover, cell types which natively express the gene of interest can be subjected to SFV infections and the feasibility of gene delivery and expression capacity can initially be evaluated by reporter gene (β -galactosidase, GFP) expression. Topologically different proteins have been successfully expressed from SFV vectors. For instance, cytoplasmic expression of bacterial β -galactosidase resulted in yields of 10^9 molecules per cell and the recombinant protein represented approximately 25% of the total cellular protein yields. Other intracellular proteins, which have resulted in high expression levels, are dehydrofolate reductase [11], thyroid peroxidase [13] and UDP-

gluconoryltransferase [14]. Also a few secretory proteins have been expressed, such as plasminogen activator inhibitor (PAI) [15], interleukin-12 [16] and secreted alkaline phosphatase [17]. Additionally, high level of enzyme activity was obtained for human cyclooxygenase 2 (COX-2), which resides in microsomal membranes as a membrane-associated enzyme [18].

3. G protein-coupled receptors

Probably the largest group of transmembrane proteins expressed from SFV vectors has been the GPCRs. GPCRs are the major targets for drug development today, on which a quarter of the current top 200 drugs are based [19]. The seven-transmembrane topology of GPCRs has generally made it difficult to obtain high expression levels in various expression systems. In fact, the low expression yields obtained have constituted a significant bottleneck for both drug screening activities and structural studies. In contrast, SFV with its high RNA replication capacity and its strong subgenomic promoter has favored high-level GPCR expression. Furthermore, the broad host range of SFV has significantly facilitated the choice of host cells to be applied for

Table 1
SFV-based expression of membrane proteins

Membrane protein	Host cell	Function	Expression level	Ref.
Transferrin receptor	BHK	single transmembrane	5–10% of membrane prot.	[11]
Serotonin 5-HT ₃ receptor	BHK, CHO, neurons	ligand-gated ion channel	60 pmol/mg; 2 mg/l	[27,29]
Purinoreceptor P2X1, 2, 4	BHK, CHO	ligand-gated ion channel	70–150 pmol/mg	[28]
Adenosine A1, A2a, A2b	BHK, CHO	GPCR	20–50 pmol/mg; 2 mg/l	[17]
A3	BHK, CHO	GPCR	1 pmol/mg	[51]
Adrenergic α 1b, α 2b, β 2	BHK, COS	GPCR	40–200 pmol/mg; 5 mg/l	[26]
Cannabinoid R1	BHK, CHO	GPCR	20 pmol/mg	[17]
Chemokine CCK-1, CCK-2	BHK	GPCR	300,000 receptors/cell	[17]
Dopamine D2, D3, D4	BHK, CHO, HOS	GPCR	20–50 pmol/mg	[39]
Endothelin A, B	BHK, COS	GPCR	20 pmol/mg	[52]
Galanin R1, R2, R3	BHK, CHO	GPCR	20–50 pmol/mg	[17]
Histamine H1 and H2 receptors	CHO, COS	GPCR	50–100 pmol/mg	[47]
Interleukin IL8A, IL8B	BHK	GPCR	120,000 receptors/cell	[17]
Metabotropic mGluR1	BHK, CHO, neurons	GPCR	120 pmol/mg; 3–6 mg/l	[17]
2, 3, 4, 8	BHK, CHO, neurons	GPCR	9–20 pmol/mg	[48,49]
Neurokinin receptors 1, 2	BHK, CHO, HOS	GPCR	40–100 pmol/mg; 10 mg/l	[21,25]
Olfactory receptors	BHK, HEK293, OLF442	GPCR	10–20% of membrane prot.	[22]
Opioid receptors δ , κ , μ	BHK, CHO, HEK293, C6	GPCR	20–50 pmol/mg; 1–3 mg/l	[24]
Prostaglandin E2ep4	BHK, CHO	GPCR	3 pmol/mg	[53]
Serotonin 5-HT1, 6, 7	BHK, CHO, HEK293	GPCR	20–50 pmol/mg; 1–3 mg/l	[50]
Dopamine transporter	BHK	transporter	[³ H] dopamine uptake only	[34]
Cyclooxygenase 1, 2	BHK, CHO	membrane-associated	4000 pg PGE ₂ /μg micros. prot.	[18]
Catechol- <i>o</i> -methyltransferase	BHK, neurons	membrane-bound	10–20% of total prot.	[32]
Presinilin 1, 2	BHK, neurons	transmembrane protein	localization studies only	[54]
APP	BHK, CHO, neurons	amyloid precursor protein	20% of total prot.	[55]
GABA _A receptor	BHK, neurons	heteromeric channel	0.5–2 pmol/mg	[30]
Potassium channel Kv 1.1, 1.2	CHO	octomeric channel	functional studies only	[31]
Yeast syntaxin Sso2p	BHK	membrane insertion	localization studies only	[33]
G proteins: G α q, G β 2, G γ 2	BHK, COS	membrane-associated	functional coupling to GPCRs	[46]

pmol/mg, picomoles of receptor expressed per milligram of membrane protein.

mg/l, milligrams of receptor per liter of cultured cells.

prot., protein.

micros., microsomal.

specific receptor expression. Because the SFV vectors have been subjected to large-scale production of recombinant proteins in suspension cultures of mammalian cell lines, it has allowed expression of large quantities of GPCRs.

Various types of GPCRs have been successfully expressed from SFV vectors (Table 1). In general, the expression levels obtained are much higher, often 10- to 100-fold higher than achieved with many other systems. In this context, saturation binding has demonstrated B_{\max} values of 50–150 pmol receptor/mg protein for several GPCRs [20]. Binding assays on intact BHK and CHO cells suggested receptor densities of $>3 \times 10^6$ receptors per cell [21]. SFV-based GPCR expression resulted in measurable coupling to G proteins, which could be assayed for various receptors by intracellular Ca^{2+} release, inositol phosphate accumulation, cAMP stimulation and GTP γ S binding [20]. Because of the high expression levels obtained, it was reasonable to expect that not all receptors are inserted into the plasma membrane. For instance, localization studies of tagged olfactory receptors indicated that not all receptors were transported to the cell surface [22]. Similar observations were made for the human neurokinin-1 receptor (NK1R), where a biotin tag was engineered at the C terminus [17]. In this case, the biotin tag was demonstrated not to interfere with the functional activity of the NK1R measured by saturation binding and coupling to G proteins (intracellular Ca^{2+} release) [23]. Moreover, the choice of host cells played a significant role, because no signal was detected for olfactory receptors in the plasma membrane of BHK cells, whereas strong receptor expression was observed in the olfactory epithelial cell line OLF442 and in primary hippocampal neurons on the cell surface. Also, expression levels varied in different host cells. For instance, the expression levels were 2- to 5-fold higher for the rat κ and μ opioid receptors in rat C6 glioma cells compared to BHK or CHO cells [24].

Generally, SFV-based expression has generated reasonably homogenous glycosylation of GPCRs based on SDS-PAGE analysis. For instance, metabolic labeling with [^{35}S] methionine showed a single glycosylated form of NK1 [21] and NK2 [25] receptors expressed in various host cell lines. In contrast, overexpression of the hamster α_{1B} adrenergic receptor generated structural heterogeneity, which was due to N-linked glycosylation and phosphorylation, but not palmitoylation or O-linked glycosylation [26]. The heterogeneous glycosylation pattern was not a typical feature of the high-level SFV expression, but was also seen for the α_{1B} adrenergic receptor when other transient mammalian expression systems were applied.

4. Ion channels

In addition to GPCRs, ligand-gated ion channels have also been efficiently expressed from SFV vectors (Table 1). The mouse serotonin 5-HT $_3$ receptor [27] and various rat and human P2X purinoreceptors [28] demonstrated high binding

activity in several mammalian host cells. Moreover, functional activity of P2X receptors was obtained by measuring Ca^{2+} uptake in CHO cells after stimulation with ATP [29]. Additionally, the high infection rate of mammalian cells allowed efficient patch-clamp recordings on whole cells [27,28]. Overexpression of homopentameric ion channels, such as the 5-HT $_3$ receptor, has been shown to be fairly straightforward. However, for channels that require the assembly of several different subunits, it has turned out to be more complicated to achieve high specific binding activity. For instance, assembly of functional GABA $_A$ receptors required co-expression of at least α and β subunits both in BHK cells and primary neurons [30]. It was demonstrated that homomeric subunits were retained in the endoplasmic reticulum, whereas heteromeric expression resulted in receptor transport to the plasma membrane. In addition to ligand-gated ion channels, potassium channels have also been expressed from SFV vectors [31]. Functional assembly of voltage-sensitive potassium channels from Kv1.1 or 1.2 α subunits with either 1.1 or 1.2 β subunits was demonstrated in CHO-K1 cells co-infected with SFV vectors. Furthermore, co-expression of His-tagged Kv α 1.2 and β 2.1 subunits allowed purification of a 405-kDa complex, suggesting the reconstruction of an octomeric structure.

5. Other transmembrane proteins

The feasibility of transmembrane protein expression from SFV vectors was first demonstrated by overexpression of the transferrin receptor in BHK cells [11]. The expression levels were relatively high, although clearly lower than what had been obtained for intracellular reporter proteins such as bacterial β -galactosidase. Other membrane-inserted proteins, such as catechol-*o*-methyltransferases (COMT), were expressed from SFV vectors and the membrane localization could be demonstrated in both BHK cells and primary cultured superior cervical ganglion neurons [32]. Furthermore, the yeast syntaxin Sso2p demonstrated appropriate insertion into the membrane of BHK cells after expression from SFV vectors [33]. SFV-based expression of a dopamine transporter was also conducted, although only by transfection of in vitro transcribed RNA and not by infection with recombinant SFV particles [34]. For this reason, the expression levels were moderate. The molecular mass of the recombinant transporter was 56 kDa. Tunicamycin treatment reduced the size to 50 kDa, which indicated that a proper glycosylation pattern occurred. Moreover, the glycosylated transporter was localized to the plasma membrane, whereas the unglycosylated form was retained in the cytoplasm.

6. Large-scale production and purification

To facilitate the production of large quantities of recombinant proteins in cells infected with SFV vectors,

adaptation to growth in suspension cultures has been established (Fig. 2). Several mammalian cell lines such as BHK-21, CHO-K1, HEK293 and rat C6 glioma cells were successfully cultured in serum-free medium. It was demonstrated that the infection rates and expression levels were comparable to what had earlier been achieved for adherent cells [18]. Production of COX2 at 1 l scale in BHK cells cultured in spinner flasks generated high expression levels (16 mg COX2/l) with high specific enzymatic activity (3942 ± 765 pg PGE₂/microgram microsomal protein/5 min) [18]. Interestingly, the culture temperature had a significant effect on expression levels of firefly luciferase in rodent cells, showing 10- to 20-fold higher activity at 33 °C compared to 37 °C in BHK and CHO cells [35]. In contrast, the luciferase activity was only marginally higher in human HEK293 cells and no differences were obtained in HEK293(EBNA) cells.

Recombinant protein production has also been carried out in bioreactors. The mouse serotonin 5-HT₃ receptor was expressed in BHK cells cultured in 11.5 l volumes in bioreactors [29]. A relatively high multiplicity of infection (MOI) was used to enhance expression levels through multiple infections as was earlier demonstrated for β -galactosidase

expression [18]. In fermentor cultures, the best results of expression were obtained by performing SFV infections at pH 6.9 for 2 h at 37 °C and then raising the pH to 7.4 for the expression phase [36]. The BHK cells were harvested at 16–18 h post infection, at which point saturation binding determinations demonstrated 50–60 pmol receptor/mg protein, equivalent to $>3 \times 10^6$ receptors/cell. The production of batches was highly reproducible with yields of the 5-HT₃ receptor in the range of 1–2 mg/l culture.

In an attempt to further increase the expression levels of the human NK1R, a fusion to the full-length SFV capsid sequence was engineered. The capsid gene contains a translation enhancement signal at its 5' end, which is responsible for 5- to 10-fold increase in expression levels [37]. Moreover, it was demonstrated that several recombinant proteins were efficiently cleaved off by the autocatalytic cleavage activity of the capsid. The capsid–NK1R fusion generated 5- to 10-fold higher expression levels compared to individually expressed NK1 receptor estimated from metabolic labeling experiments, but also saturation binding assays [21]. When the capsid–NK1R construct was subjected to large-scale expression in spinner flasks, yields of 5–10 mg receptor/l culture were achieved.

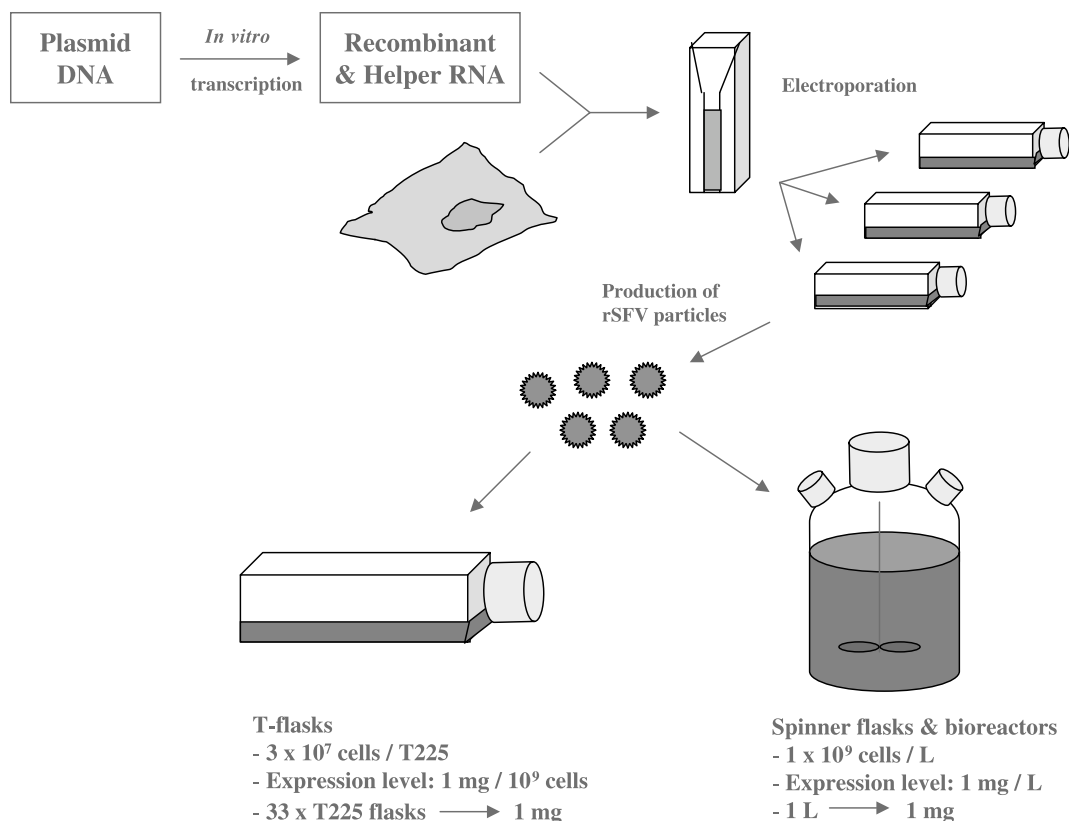


Fig. 2. Large-scale production in adherent cells and suspension cultures. Recombinant SFV particles are generated from plasmid DNA by in vitro transcription of RNA and co-electroporations. Larger volumes of SFV particles are produced by electroporations in parallel cuvettes and/or larger cuvettes and co-culturing in parallel T-flasks. Large-scale protein production can be performed in adherent cells in T-flasks or roller-bottles or in suspension cultures in spinner flasks or bioreactors.

7. Structural biology applications

The ease of engineering new SFV constructs and the rapid virus production has also favored mutagenesis studies. Site-directed mutagenesis and molecular modeling approaches have been combined to validate the involvement of 11 amino acid residues in antagonist binding of the human NK1 receptor [38]. Mutants Q165A, H197A and F268A showed significantly reduced binding affinity for some NK1 antagonists in CHO cells infected with recombinant SFV–NK1R mutant particles. The results suggested that all antagonists seemed to bind in a similar region of the receptor, but did not rely on the same binding site interactions. In another study, nine mutants and the wild-type human dopamine D3 receptor were expressed in CHO cells and analysed for binding properties with several structurally different dopamine D3 ligands [39]. Sequence alignment based on bacteriorhodopsin generated a model with amino acid residues Thr369 and Leu349 located on the inside of the binding pocket. The mutagenesis study revealed a significant decrease for the His349Leu mutation in pK_i values for raclopride, dopamine and GR218231, but an affinity increase for GR99841, whereas the Thr369Val mutation showed an increase in pK_i values for both GR99841 and 7-OH-DPAT.

Production of tens of milligrams of receptor protein in mammalian host cells has not only served drug screening applications, but also studies in structural biology. Efficient solubilization conditions were established for the overexpressed 5-HT₃ receptor, where the detergent C₁₂E₉ showed only minor effects on the receptor binding activity [40]. The C-terminally hexa-histidine tagged receptor was subjected to a one-step purification by metal ion affinity chromatography and this resulted in visualization of a single band with a mobility of 65 kDa in SDS-PAGE. Treatment with peptide *N*-glycosidase F led to reduction of the molecular mass to 49 kDa, suggesting an appropriate glycosylation of the recombinantly expressed receptor. Size exclusion chromatography was applied to determine the molecular mass of the functional receptor complex of 280 kDa, which is in good agreement with a pentameric structure. Dialyzed purified receptor preparations were subjected to circular dichroism to determine the secondary structure revealing mainly α -helices (50%) and β -strands (24%).

8. MePNet, structural genomics on GPCRs

Even though high expression levels of transmembrane proteins have been achieved with the SFV system, there are major obstacles before high-resolution structures can be obtained. Much attention needs to be paid to improvements in the areas of solubilization, purification, stabilization and crystallization. However, the feasibility of GPCR crystallization was demonstrated recently by the successful determination of a high-resolution structure for bovine rhodopsin

[41], although this photoreceptor was isolated from bovine retina and not expressed as a recombinant protein.

One approach to allow statistical analysis of the expression of a large number of GPCRs and also to develop various technologies from expression to crystallization has been the introduction of the Membrane Protein Network (MePNet) structural genomics program [42]. MePNet is a network consisting of four academic partners and is supported by more than 40 biotech and pharmaceutical companies worldwide. The goal of the program is to overexpress 100 GPCRs in three expression systems based on *E. coli*, *P. pastoris* and SFV vectors. The 30 best expressed GPCRs in bacterial inclusion bodies will be subjected to refolding exercises before purification attempts. In parallel, the 15 best overexpressed GPCRs in yeast and mammalian cell membranes will be subjected to solubilization studies. Successful refolding/solubilization will provide the basis for future purification and crystallization attempts. This 3-year research project aims at improving existing technologies, which already within this period might generate novel high-resolution structures for GPCRs.

9. Conclusions and future aspects

SFV vectors have proven useful for the overexpression of various integral transmembrane proteins. The rapid high-titer virus production and broad host range have made these vectors attractive for versatile applications in mammalian cell lines and primary cell cultures. The high authenticity of recombinantly expressed proteins is one of the most appreciated assets of using SFV vectors. Large-scale production of GPCRs and ligand-gated ion channels in serum-free suspension cultures of several mammalian cell lines has substantially facilitated purification efforts. Needless to say, large-scale use of viral vectors always needs to take into consideration the safety risks related to viral infectivity and pathogenicity. The SFV vectors described in this review are replication deficient. Additionally, the use of the second-generation SFV helper vector (pSFV-Helper2) certifies the production of only conditionally infectious particles due to the presence of three point mutations in the SFV structural genes [43]. Moreover, handling of SFV-infected cells and other material has been demonstrated to be safe. Already simple washes of cell cultures efficiently remove any residual infectious particles [44]. Recombinant proteins expressed from SFV vectors can therefore be safely removed from the cell culture facility for activity assays and downstream processing.

As for all research tools, technology improvements are essential also for SFV vectors and their applications. Novel less cytotoxic and temperature-sensitive mutant vectors have recently been developed [44,45]. These should allow a longer survival of host cells, which should be an advantage for establishing a prolonged expression phase with potentially higher recombinant protein yields. The co-

expression of GPCRs and G protein subunits is an interesting application of the SFV system. It was shown that co-infection of COS-7 cells with SFV particles carrying the α_{1B} adrenergic receptor and the $G\alpha_q$, $G\beta_2$ and $G\gamma_2$ subunits enhanced significantly the functional coupling activity [46]. Co-expression could be a potentially attractive future approach to stabilize GPCRs for purification and crystallization.

References

- [1] K. Murata, K. Mitsuoka, T. Hirai, T. Walz, P. Agre, J.B. Heymann, A. Engel, Y. Fujiyoshi, *Nature* 407 (2000) 599–605.
- [2] G.J. Turner, R. Reusch, A.M. Winter-Vann, L. Martinez, M.C. Betlach, *Protein Expr. Purif.* 17 (1999) 312–323.
- [3] A. Hinnen, F. Buxton, B. Chaudhuri, J. Heim, T. Hottiger, B. Meyhack, G. Pohlig, in: A. Smith (Ed.), *Gene Expression in Recombinant Microorganisms*, Marcel Dekker, New York, 1994, pp. 121–193.
- [4] P. Sander, S. Grunewald, H. Reilander, H. Michel, *FEBS Lett.* 344 (1994) 41–46.
- [5] H.M. Weiss, W. Haase, H. Michel, H. Reilander, *Biochem. J.* 330 (1998) 1137–1147.
- [6] K.E. Mazina, C.D. Strader, T.M. Fong, *J. Recept. Res.* 14 (1994) 63–73.
- [7] C. Xiong, R. Levis, P. Shen, S. Schlesinger, C.M. Rice, H.V. Huang, *Science* 243 (1989) 1188–1191.
- [8] N.L. Davis, K.W. Brown, R.E. Johnston, *Virology* 171 (1989) 189–204.
- [9] K. Lundström, *Intervirology* 43 (2000) 247–257.
- [10] K. Lundström, *Curr. Gene Ther.* 1 (2001) 19–29.
- [11] P. Liljeström, H. Garoff, *Bio/Technology* 9 (1991) 1356–1361.
- [12] K. Lundström, *J. Recept. Signal Transduct. Res.* 19 (1999) 673–686.
- [13] H. Bikker, F. Bass, J.-J.M. de Vijlder, *J. Clin. Endocrinol. Metab.* 82 (1997) 649–653.
- [14] T. Forsman, P. Lautala, K. Lundström, K. Monastyrskaya, M. Ouzine, B. Burchell, J. Taskinen, I. Ulanen, *Life Sci.* 67 (2000) 2473–2484.
- [15] J.L. Chuang, R.R. Schleef, *Cell Biochem.* 82 (2001) 277–289.
- [16] J. Zhang, C. Asselin-Paturel, F. Bex, J. Bernard, J. Chehimi, F. Willem, A. Caignard, P. Berglund, P. Liljeström, A. Burny, S. Chouaib, *Gene Ther.* 4 (1997) 367–374.
- [17] K. Lundström, unpublished results.
- [18] H.D. Blasey, K. Lundström, S. Tate, A.R. Bernard, *Cytotechnology* 24 (1997) 65–72.
- [19] W.J. Bailey, et al., *Exp. Opin. Ther. Patents* 11 (2001) 1861–1887.
- [20] K. Lundström, C. Schweitzer, D. Rotmann, D. Hermann, E.M. Schneider, M.U. Ehrenguber, *FEBS Lett.* 504 (2001) 99–103.
- [21] K. Lundström, A. Mills, G. Buell, E. Allet, N. Adami, P. Liljeström, *Eur. J. Biochem.* 224 (1994) 917–921.
- [22] K. Monastyrskaya, F. Goepfert, R. Hochstrasser, G. Acuna, J. Leighton, J.R. Pink, K. Lundström, *J. Recept. Signal Transduct. Res.* 19 (1999) 687–701.
- [23] K. Lundström, A. Vargas, B. Allet, *Biochem. Biophys. Res. Commun.* 208 (1995) 260–266.
- [24] K. Lundström, R. Henningsen, *J. Neurochem.* 71 (Suppl. 1) (1998) S50D.
- [25] K. Lundström, A. Mills, E. Allet, K. Ceszkowski, G. Agudo, A. Chollet, P. Liljeström, *J. Recept. Signal Transduct. Res.* 15 (1995) 23–32.
- [26] K. Björklöf, K. Lundström, L. Abuin, P.J. Greasley, S. Cotecchia, *Biochemistry* 41 (2002) 4281–4291.
- [27] P. Werner, E. Kawashima, J. Reid, N. Hussy, K. Lundström, G. Buell, Y. Humbert, J.A. Jones, *Mol. Brain Res.* 26 (1994) 233–241.
- [28] A.D. Michel, K. Lundström, G. Buell, A. Surprenant, S. Valera, P.P.A. Humphrey, *Br. J. Pharmacol.* 118 (1996) 1806–1812.
- [29] K. Lundström, A. Michel, H. Blasey, A.R. Bernard, R. Hovius, H. Vogel, A. Surprenant, *J. Recept. Signal Transduct. Res.* 17 (1997) 115–126.
- [30] G.H. Gorrie, Y. Vallis, A. Stephenson, J. Whitfield, B. Browning, T.G. Smart, S.J. Moss, *J. Neurosci.* 17 (1997) 6587–6596.
- [31] O. Shamotienko, S. Akhtar, C. Sidera, F.A. Meunier, B. Ink, M. Weir, J.O. Dolly, *Biochemistry* 38 (1999) 16766–16776.
- [32] I. Ulanen, J. Peränen, J. Tenhunen, C. Tilgmann, T. Karhunen, P. Panula, L. Bernasconi, J.-P. Aubry, K. Lundström, *Eur. J. Biochem.* 243 (1997) 452–459.
- [33] J. Jäntti, S. Keränen, J. Toikkanen, E. Kuusimäki, C. Ehnholm, H. Söderlund, V.M. Olkkonen, *J. Cell Sci.* 107 (1994) 3623–3633.
- [34] T. Lenhard, K. Marheineke, B. Lingen, W. Haase, R. Hammernann, H. Michel, H. Reiländer, *Cell. Mol. Neurobiol.* 18 (1998) 347–360.
- [35] E.-J. Schlaeger, K. Lundström, *Cytotechnology* 28 (1998) 205–211.
- [36] H.D. Blasey, B. Brethon, R. Hovius, H. Vogel, A.-P. Tairi, K. Lundström, L. Rey, A.R. Bernard, *Cytotechnology* 32 (2000) 199–208.
- [37] M. Sjöberg, M. Suomalainen, H. Garoff, *Bio/Technology* 12 (1994) 1127–1131.
- [38] K. Lundström, A.B. Hawcock, A. Vargas, P. Ward, P. Thomas, A. Naylor, *Eur. J. Pharmacol.* 337 (1997) 73–81.
- [39] K. Lundström, M.P. Turpin, C. Large, G. Robertson, P. Thomas, X.-Q. Lewell, *J. Recept. Signal Transduct. Res.* 18 (1998) 133–150.
- [40] R. Hovius, A.-P. Tairi, H. Blasey, A. Bernard, K. Lundström, H. Vogel, *J. Neurochem.* 70 (1998) 824–834.
- [41] K. Palczewski, T. Kumasaki, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Tong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, M. Miyano, *Science* 289 (2000) 739–745.
- [42] K. Lundström, Structural genomics on GPCRs: the MePNet approach, *Curr. Drug Discov. Mag.* (2002) 29–33.
- [43] P. Berglund, M. Sjöberg, H. Garoff, G.J. Atkins, B.J. Sheahan, P. Liljeström, *Bio/Technology* 11 (1993) 916–920.
- [44] K. Lundström, D. Rotmann, D. Hermann, E.-J. Schlaeger, *Cytotechnology* 35 (2001) 213–221.
- [45] K. Lundström, D. Rotmann, D. Hermann, E.M. Schneider, M. Ehrenguber, *Histochem. Cell Biol.* 115 (2001) 83–91.
- [46] A. Scheer, K. Björklöf, S. Cotecchia, K. Lundström, *J. Recept. Signal Transduct. Res.* 19 (1999) 369–378.
- [47] M. Hoffmann, D. Verzijl, K. Lundström, U. Simmen, A. Alewijnse, H. Timmerman, R. Leurs, *Eur. J. Pharmacol.* 427 (2001) 105–114.
- [48] C. Schweitzer, C. Kratzeisen, G. Adam, K. Lundström, P. Malherbe, S. Ohresser, H. Stadler, J. Wichmann, T. Woltering, V. Mutel, *Neuropharmacology* 39 (2000) 1700–1706.
- [49] P. Malherbe, C. Kratzeisen, K. Lundström, J.G. Richards, R.L.M. Faull, V. Mutel, *Mol. Brain Res.* 67 (1999) 201–210.
- [50] U. Simmen, J. Higelin, W. Schaffner, K. Lundström, *Pharmacopsychiatry* 34 (Suppl. 1) (2001) S137–S142.
- [51] M. Patel, C. Harris, K. Lundström, *Drug Dev. Res.* 40 (1997) 35–40.
- [52] H. Cramer, K. Schmenger, K. Heinrich, H. Boening, A. Breit, A. Piiper, C. Liebmann, K. Lundström, W. Mueller-Esterl, C. Schroeder, *Eur. J. Biochem.* 268 (2001) 5449–5459.
- [53] F. Marshall, K. Patel, K. Lundström, J. Camacho, S.M. Foord, M.G. Lee, Characterization of [3 H]-prostaglandin E_2 binding to prostaglandin EP_4 receptor expressed with Semliki Forest virus, *Br. J. Pharmacol.* 121 (1997) 1673–1678.
- [54] D.G. Cook, J. Sung, T. Golde, K. Felsenstein, B.S. Wojczyk, R.E. Tanzi, J.Q. Trojanowski, V.M.-Y. Lee, R.W. Doms, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 9223–9228.
- [55] M. Simons, P. Tienari, C.G. Dotti, K. Beyreuther, *FEBS Lett.* 368 (1995) 363–366.