Minireview

Semliki Forest virus vectors: efficient vehicles for in vitro and in vivo gene delivery

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Abstract Rapidly generated high-titer Semliki Forest virus (SFV) vectors can infect numerous mammalian cell lines and primary cell cultures, and result in high levels of transgene expression. SFV-based expression of transmembrane receptors has been characterized by specific ligand-binding activity and functional responses. Adaptation of the SFV technology for mammalian suspension cultures has allowed the production of hundreds of milligrams of recombinant receptor for purification and structural studies. The same SFV stock solutions used for the infection of mammalian cells in culture have also been successfully applied for efficient transgene expression in organotypic hippocampal slices, as well as in vivo in rodent brain.

Key words: Semliki Forest virus vector; Recombinant receptor; Protein purification; Neuronal expression; In vivo gene delivery; Hippocampus; Connexin 36

1. Introduction

Recombinant protein expression is today a prerequisite for basic research and modern drug discovery. The completion of the sequencing of the human genome has further accelerated the demand for rapid and efficient production of recombinant protein. It is therefore no surprise that intensive development on various expression systems has resulted in novel and improved gene delivery vectors. A major issue has been the expression system to be used. It has become evident that there is no universal perfect system applicable to each recombinant protein of interest. Expression vectors developed for *Escherichia coli*, yeast and insect cells have shown high expression of many recombinant proteins. However, more complex mammalian proteins require specific factors and chaperone proteins for correct folding and transport, and are subjected to different post-translational modification processes. In attempts to circumvent these problems for non-mammalian expression vectors, defined epitopes and transmembrane regions have been deleted or modified. Alternatively, only extracellular domains have been expressed or fusions made to well-expressed bacterial proteins. The use of mammalian expression vectors to produce functional proteins, by contrast, is another approach. Unfortunately, mammalian expression vectors have generally resulted in low recombinant protein yields and, additionally, have been time-consuming and labor-intensive to use.

Replication-deficient alphavirus vectors, specifically Semliki Forest virus (SFV) and Sindbis virus vectors, have become attractive alternatives for rapid and high-level gene delivery [1,2]. Alphavirus vectors were engineered to allow the expression of topologically different proteins in a broad range of mammalian host cells [3]. Here, we review various applications of SFV vectors for both in vitro and in vivo gene delivery. We describe the use of SFV vectors for the expression of several G protein-coupled receptors (GPCRs) and ligand-gated ion channels. Because of their strong preference for neuronal infection in the central nervous system, SFV vectors are favorable for expression and localization studies in dispersed primary neurons in culture, as well as in organotypic hippocampal slices. Moreover, SFV vectors efficiently deliver genes to the rat brain in vivo, and preliminary studies in animal tumor models have generated encouraging anti-tumor responses. We also summarize the development of novel, improved SFV vectors.

2. Expression of recombinant receptors in cell lines

SFV vectors have been applied for the expression of >50 different GPCRs [4]. In many instances, extremely high levels of expression have been achieved, as measured by specific binding activity on intact cells or isolated membranes. B_{max} values of up to 150 pmol receptor/mg protein, and receptor densities of $>5\,000\,000$ receptors/cell have been obtained. SFV-mediated GPCR expression in various cell lines showed some variation in expression levels, which interestingly depended on the time of post-infection (Fig. 1). For instance, expression of the human neurokinin-1 receptor (hNK1R) in Chinese hamster ovary (CHO) cells demonstrated maximal specific agonist-binding activity at 12 h post-infection [5], whereas baby hamster kidney (BHK) cells infected with SFV vectors carrying the hamster α_{1b} adrenergic receptor

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Abbreviations: BHK, baby hamster kidney; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; hNK1R, human neurokinin-1 receptor; SFV, Semliki Forest virus

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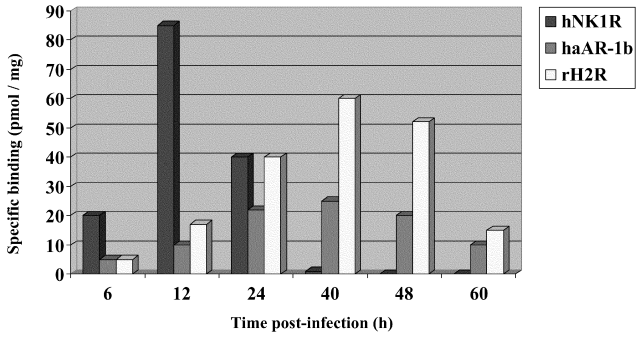

Fig. 1. Time-dependent SFV-mediated expression of GPCRs in mammalian host cells. BHK-21, CHO-K1 and COS-7 cells were infected with SFV vectors encoding the human neurokinin-1 receptor (hNK1R), the hamster α_{1b} adrenergic receptor (haAR-1b) and the rat histamine H2 receptor (rH2R), respectively. Saturation binding on isolated membranes was performed with [^{3}H]substance P (hNK1R) [4], [^{125}I]HEAT (haAR-1b) [6] and [^{125}I]iodoaminopotentidine (rH2R), as previously described [7].

showed a much later peak of expression, i.e. at 40 h post-infection [6]. Likewise, the highest levels of rat histamine H2 receptor expression in COS-7 cells were reached at 40 h after infection [7]. The different expression patterns mainly corresponded to the individual features of the expressed GPCRs, although some variations in expression levels occurred between different host cell lines.

Functional coupling of SFV-expressed GPCRs to G proteins has been demonstrated by measuring intracellular Ca^{2+} release, inositol triphosphate accumulation, cAMP stimulation, and GTPγS binding. As described for the agonist-binding activity, functional responses also followed a time-dependent expression pattern [5], most likely due to SFV-induced inhibition of host cell protein synthesis and apoptosis. Additionally, the unfavorable ratio between exogenous receptor and endogenous G proteins, due to the extreme receptor overexpression and inhibition of endogenous G protein synthesis, contributes to the decrease in functional activity observed with time. The limited amount of endogenous G proteins, however, can be compensated for by SFV-mediated co-expression of G protein subunits [6].

SFV vectors have also been applied for the expression of ligand-gated ion channels [8,9]. Extreme levels of specific agonist-binding activity were obtained for the mouse serotonin 5-HT$_3$ receptor and the rat and human p2X purine receptors. Additionally, functional ATP-induced responses were demonstrated for these channels by electrophysiological recordings [8,9] and Ca^{2+} measurements [10].

The adaptation of SFV technology to mammalian cells in suspension cultures has significantly facilitated the production of large quantities of recombinant receptors [11]. Infection of BHK cells in a bioreactor of 11.5 l resulted in the production of 20 mg 5-HT$_3$ receptor (B_{max} value of 52 pmol receptor/mg protein, 3.2×10^6 receptors/cell) [10]. The production was highly reproducible (yields of 1–2 mg receptor/l) and allowed efficient purification and preliminary characterization of the C-terminal hexa-histidine-tagged 5-HT$_3$ receptor [12]. The purification of the receptor, verified by SDS–PAGE, yielded a single 65 kDa polypeptide, which is in good agreement with the postulated size of the glycosylated 5-HT$_3$ receptor subunit. Deglycosylation reduced the size to 49 kDa. Furthermore, the purified receptor showed a specific ligand-binding activity of

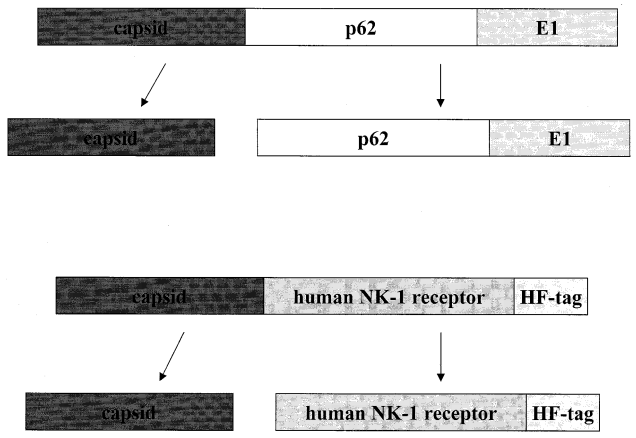

Fig. 2. SFV capsid fusion protein for enhancement of recombinant protein expression. The presence of a translation-enhancement signal in the capsid sequence results in increased expression levels of SFV p62 and E1 polyproteins. Furthermore, the capsid protein is autocatalytically cleaved from the fusion protein. Similarly, the fusion of the full-length capsid sequence to the hNK1R enhances transgene expression by five- to 10-fold, and results in the efficient cleavage of the capsid-hNK1R fusion protein. HF-tags, FLAG-tag and hexa-histidine tag.

$\sim$5 nmol/mg protein for [^{3}H]GR65630. Secondary structure determinations by circular dichroism indicated mainly α-helices (50%) and β-strands (24%), and minor contributions from non-regular structures (9%). Size exclusion chromatography suggested a molecular mass of 280 kDa for the functional receptor complex, which is in good agreement with the postulated pentameric structure. Preliminary cryo-electron microscopy studies on the purified 5-HT$_3$ receptor also suggested a channel structure consisting of five subunits.

To further improve the receptor-expression capacity of SFV vectors, a fusion construct to the SFV capsid gene was engi-

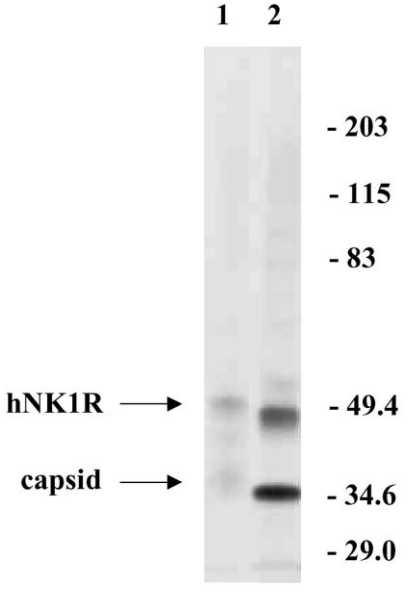

Fig. 3. Metabolic labeling of BHK-21 cells infected with recombinant SFV. BHK-21 cells were infected with SFV vectors expressing the hNK1R (lane 1) or the hNK1RHF as a capsid fusion (lane 2), and labeled with [^{35}S]methionine at 16 h post-infection. Cell lysates were subjected to 10% SDS–PAGE and visualized by autoradiography.

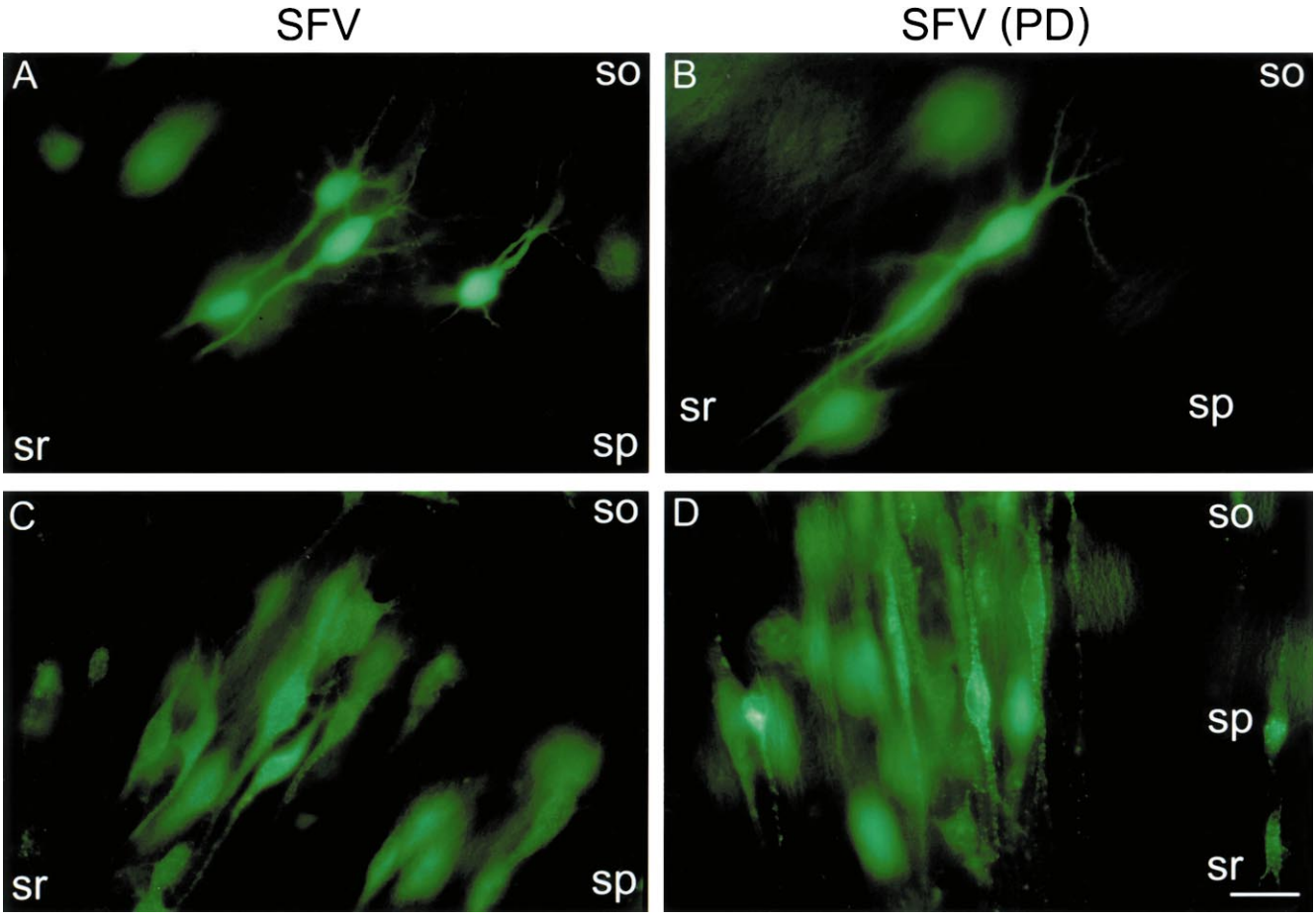

Fig. 4. Recombinant SFV-mediated gene transfer into CA1 pyramidal cells of cultured rat hippocampal slices. A,B: Expression of GFP, as revealed by fluorescence microscopy, in slices fixed at 14 days in culture and 2 days after injection of 100-fold diluted wild-type SFV-GFP (A) and 10-fold diluted SFV(PD)-GFP (B). C,D: Expression of rat connexin 36 fused to GFP (Cx36-GFP) in slices fixed at 14–15 days in culture and 2 days after injection of wild-type SFV-Cx36-GFP (C) and 3 days after injection of SFV(PD)-Cx36-GFP (D). Note the punctuate GFP fluorescence staining pattern in infected cells. Abbreviations: so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; scale bar = 40 μm.

neered. Similar to the Sindbis virus [13], it has been reported earlier that the 5′-end of the capsid gene contains a translation-enhancement signal, which can increase transgene expression levels by five- to 10-fold [14]. Upon fusing the full-length capsid sequence to the transgene, it was observed that recombinant proteins were efficiently cleaved off by the autocatalytic cleavage activity of the capsid protein. To enhance the expression levels of the hNK1R, a fusion to the full-length capsid sequence was engineered (Fig. 2). A hexa-histidine-tag and a FLAG-tag were introduced at the C-terminus to facilitate receptor purification and identification, respectively. The generated SFV construct yielded approximately five-fold higher receptor levels when compared to the hNK1R expressed from the SFV vector lacking the capsid sequence [4]. Furthermore, the fusion protein was efficiently cleaved into the capsid and hNK1R proteins (Fig. 3). Large-scale production of hNK1R in serum-free CHO cell suspension cultures resulted in 5–10 mg of receptor protein/l culture. Cell-fractionation studies revealed that although the majority (80%) of the recombinant receptors was located in internal structures, the receptors showed functional binding of hNK1R ligands. The purification of hundreds of milligrams of hNK1R has been achieved and preliminary structural studies are in progress.

3. Gene delivery to neurons in primary cultures and hippocampal slices

Alphavirus vectors have generally shown a strong preference for infection of neurons [15]. Recombinant SFV particles have therefore been used to efficiently deliver genes into dispersed primary neurons in culture [16]. The infection rate is impressively high and ranges between 75 and 95%. Even when primary hippocampal neurons were cultured on a feeder layer of glial cells, >90% of the infected cells were neurons [17]. SFV-infected neurons were intact for several days post-infection, and SFV-mediated expression of metabotropic glutamate receptors resulted in the functional inhibition of voltage-gated calcium currents [18].

Similar to cultures of dissociated cells, SFV vectors both efficiently and preferentially infect neurons in organotypic slices prepared from the hippocampus of 6-day-old rats [19,20]. At 1–2 days post-infection, >90% of the transduced cells were either pyramidal cells or interneurons. Similar results were obtained with the closely related Sindbis virus [19,21]. Fig. 4 shows an example of CA1 pyramidal cells expressing soluble green fluorescent protein (GFP; top panels) and a GFP fusion of the gap-junction-forming membrane protein connexin 36 (bottom panels). For both transgenes, the wild-

type SFV vector and the less cytotoxic SFV(PD) mutant (see below) were used. As expected, the observed GFP staining pattern is similar for both forms of the SFV vector. The time course of GFP expression in cultured slices, however, was transient and showed a peak at 1–2 days post-infection for both wild-type SFV and SFV(PD) [20]. Present forms of SFV are therefore useful for short- rather than long-term gene expression. In any case, cell-viability experiments showed that electrophysiological properties of SFV-infected neurons seem normal at 2 days post-infection [20], and $>90\%$ of the infected cells appear intact at 5 days post-infection, as they exclude propidium iodide [19]. Compared to other, more commonly used, viral vectors derived from adenovirus, adeno-associated virus, and lentivirus, recombinant SFV is preferred for rapid, high-level and neuron-specific transgene expression [20].

4. Gene delivery to specific brain regions

One advantage of alphavirus vectors is that stocks of the same replication-deficient virus particles used to infect cell lines in culture can also be applied in vivo without any additional purification or concentration. Sindbis virus expressing β-galactosidase injected into the nucleus caudata/putamen and nucleus accumbens septi of mice resulted in a transient reporter gene expression [22]. Similarly, injection of SFV-LacZ particles into the striatum and amygdala of rat brain caused a local, high-level β-galactosidase expression [23]. The viral injection had no impact on the general behavior of the rats up to 28 days post-injection, as monitored by food intake, body weight, body temperature, muscle strength, sensorimotor function, and exploratory behavior. Similar to our data in cultured hippocampal slices, maximal transgene expression was obtained at 1–2 days post-injection, and β-galactosidase levels declined thereafter. In situ hybridization data confirmed the transient nature of gene expression. Obviously, alphaviral vectors are therefore not suitable, at least in their present form, for studies where long-term expression is required. In contrast, short-term gene expression can be desirable in some applications, e.g. when the effect of gene expression on behavior has to be monitored before, during, and after the overexpression of the protein.

5. Animal models for cancer gene therapy

Because SFV vectors, as described above, have demonstrated a transient expression pattern both in vitro and in vivo, they can be considered as potentially attractive for cancer gene therapy applications. In this context, favorable features of the SFV system are the enormous RNA replication in the cytoplasm that results in extreme transgene expression levels, and the ability to induce apoptosis in infected cells, which has been seen in rat glioma and gliosarcoma cell lines [24], as well as in human prostate tumor cell lines [25]. Recently, SFV vectors have been injected intratumorally to study therapeutic efficacy in two mouse tumor models. SFV vectors expressing both the p40 and p35 subunits of interleukin-12 (IL-12) from the same vector, using two subgenomic SFV 26S promoters, were injected into a B16 mouse melanoma model and monitored for tumor regression by Doppler ultrasonography [26]. SFV-mediated IL-12 expression resulted in significant tumor regression and inhibition of tumor blood vessel formation. Moreover, repeated injections resulted in enhanced anti-tumor response, but did not elicit any SFV-related antibody response. In another study, empty SFV vectors, or vectors expressing reporter genes (SFV-LacZ and SFV-GFP) were injected into human lung carcinoma that had been implanted into nude mice [27]. Significant tumor regression was observed after injecting SFV vectors on three consecutive days, followed by another three injections a week later.

6. Vector development

Although SFV vectors have a broad application range, there are two features that limit, to some extent, their use. First, the transient nature of expression, due to the instability of the viral genomic RNA molecules, is a drawback if long-term expression is desired. Prolongation of expression could also improve the expression yields, particularly for secreted proteins. Second, the high cytotoxicity of SFV vectors for host cells will to some extent limit transgene expression due to premature cell death. The dramatic shut-down of host cell protein synthesis and the induction of apoptosis certainly restricts the host cell viability after SFV infections. This effect clearly limits studies on expression kinetics and signal transduction pathways.

For this reason, less cytotoxic alphavirus vectors have been developed. A single point mutation in the non-structural protein gene nsP2 of Sindbis virus resulted in a non-cytopathogenic phenotype [28]. Unfortunately, this phenotype was observed only in very few cell lines, and viral RNA replication and expression levels were reduced, resulting in lower transgene expression. We recently reported a less cytotoxic SFV vector, SFV(PD), with two point mutations in the nsP2 gene [29]. Interestingly, compared to the wild-type SFV vector, SFV(PD) caused a seven- to 10-fold higher GFP and β-galactosidase expression, and permitted a substantially prolonged survival of many mammalian host cell lines and of dissociated hippocampal neurons. Upon infection with SFV(PD), host cell protein synthesis is only slightly affected, and the induction of apoptosis is delayed (Lundstrom, unpublished results). In addition to SFV(PD), we have engineered novel SFV vectors with both non-cytopathogenic and temperature-sensitive phenotypes [30]. These vectors showed no or reduced transgene expression at the non-permissive temperature (36–37°C), but caused extreme levels of overexpressed protein at a lower temperature (31°C). Recently, a novel mutant vector with a single point mutation in the nsP2 gene was obtained after transfecting BHK cells with SFV vectors containing randomly mutagenized SFV non-structural genes upstream of a neo-resistance gene [31]. This SFV vector was replication persistent, demonstrated very low cytotoxicity, and resulted in a significantly prolonged duration of transgene expression.

7. Conclusions

SFV vectors have now been used for various in vitro and in vivo applications. The relatively large cloning capacity (5–8 kb) [20], the easy and rapid generation of high-titer virus stocks ($>10^9$ infectious particles/ml) [32] and the broad host range have made these vectors attractive. The extremely high expression levels obtained for GPCRs and ion channels

have significantly facilitated the production of large quantities of membrane proteins. This has opened up new possibilities for advanced structural biology studies on membrane receptors. Moreover, SFV vectors have shown their high capacity to infect neurons in primary cell cultures and in brain slices, which allows the examination of gene function in the central nervous system. Recently, SFV vectors have been applied in vivo and have resulted in encouraging therapeutic responses in tumor models without detectable anti-viral side effects. Further vector development has generated novel non-cytopathogenic, as well as temperature-sensitive SFV mutants that will extend the SFV application range. Studies on expression kinetics and signal-transduction events, as well as ribozyme and antisense technologies, should now be feasible with these new SFV vectors.

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