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Novel Semliki Forest Virus Vectors with Reduced Cytotoxicity and Temperature Sensitivity for Long-Term Enhancement of Transgene Expression

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Alphaviral vectors inhibit host cell protein synthesis and are cytotoxic. To overcome these limitations, we modified the nonstructural protein-2 (nsP2) gene in the Semliki Forest virus (SFV) vector, pSFV1. Packaging of SFV replicons with two point mutations in *nsP2* resulted in high-titer recombinant SFV(PD) particles. SFV(PD) led to more efficient host cell protein synthesis, exhibited reduced cytotoxicity and improved cell survival, and allowed greater and prolonged transgene expression than the original vector, SFV. In dissociated hippocampal neurons and organotypic rat hippocampal slices, SFV(PD) infection preserved neuronal morphology and synaptic function more efficiently than SFV. Combination of the two point mutations with a replication-persistent mutation in *nsP2* resulted in a highly temperature-sensitive vector, SFV(PD713P), which efficiently transduced neurons in hippocampal slice cultures. At 31 °C, SFV(PD713P) allowed continuous transgene expression in BHK cells, at amounts comparable to SFV(PD). These new SFV mutants are expected to substantially broaden the application of alphaviral vectors in neurons and other mammalian cells.

Key Words: alphavirus, expression vector, metabolic labeling, green fluorescent protein (GFP), hippocampus, neuron, synapse staining, slice culture

INTRODUCTION

Recombinant protein expression is an essential part of modern molecular biology research and drug screening processes. Very efficient expression systems have been developed for alphaviruses, mainly Semliki Forest virus (SFV) [1] and Sindbis virus (SIN) [2]. The simple and rapid high-titer virus production and the extremely broad host cell range have permitted efficient transduction of a variety of mammalian cell lines and primary cell cultures [3]. However, a major drawback of alphaviral vectors has been their substantial inhibition of host cell protein synthesis within hours upon infection, resulting in a rapid decrease of endogenous gene expression, induction of apoptosis, and cell death within 72 hours of infection [3,4]. Early cytotoxicity has substantially restricted the applicability of alphaviral vectors and impaired their use for long-lasting experiments, such as kinetic studies or the investigations of specific signal transduction pathways during development.

The alphaviral nonstructural proteins 1–4 (nsP1–nsP4) form the cytoplasmic RNA replicase complex and play essential roles for virus function: nsP1 is required for the initiation of minus-strand RNA synthesis and the capping of viral RNAs, nsP2 contains protease and helicase activity, and nsP4 is the catalytic subunit of the viral RNA polymerase [4]. NsP3 is a phosphoprotein [5] involved in alphaviral RNA replication [6], but its precise function remains unknown. Spontaneous mutations discovered in the nonstructural genes, particularly in nsP2 and nsP4, have considerable effects on the viral pathogenicity.

Mutations in the *nsP2* gene of SIN have produced viruses [7] and replicons [7–9] with greatly reduced cytopathogenicity. The majority of the mutants had a single change at amino acid residue 726. For example, a change at this site from proline to serine produced replicons that had reduced levels of RNA replication and cytopathogenicity [7]; the change from proline to leucine led to a further reduction both in RNA synthesis and cytopathogenicity [8]. Temperature-sensitive mutations have been

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described for SIN in each of the *nsP* genes [10,11], and those in *nsP4* [12] have been introduced into SIN expression vectors. Combination of the P726S change in *nsP2* and a temperature-sensitive mutation in *nsP4* led to the inducible expression of replicons in a variety of cell lines [12].

A study on replication-competent SFV also revealed a point mutation (R649D) in the *nsP2* nuclear localization signal to confer a lower virulence in mice [13,14]. Furthermore, the introduction of known temperature-sensitive mutations into *nsP2* and *nsP4* reduced the cytotoxicity of SFV vectors [15]. In addition, random mutagenesis of *nsP* genes in SIN and SFV vectors containing the neomycin resistance gene generated replication-persistent vectors that had either deletions or point mutations in *nsP2* [9].

Here we describe (1) a vector based on the SFV4 strain, SFV(PD), harboring two point mutations in *nsP2* (S259P, R650D), as well as (2) a temperature-sensitive vector, SFV(PD713P), wherein the replication-persistent L713P mutation in *nsP2* was introduced into SFV(PD). These novel SFV vectors were found to be less cytotoxic than the original SFV vectors, and lead to higher transgene expression levels in mammalian cell lines, primary hippocampal and cortical neurons, and organotypic hippocampal slices. In addition, host cell protein synthesis, neuronal morphology and dendritic arborization, synapses, and synaptic vesicular exo- and endocytosis in infected cells were much better conserved than with original SFV vectors.

RESULTS

Effect on Host Cell Protein Synthesis

In contrast to the strong inhibition of endogenous gene expression by the SFV4-based vector, infection with SFV(PD) encoding LacZ, luciferase, and hNK1R still permitted substantial host cell protein synthesis at 16 hours post-infection in BHK and HEK 293 cells, as determined by metabolic labeling (Figs. 1A and 1B, respectively). Similar results were found in Chinese hamster ovary cells (data not shown). Likewise, in rat E18 hippocampal neurons, wherein infection with SFV-LacZ produced a substantial inhibition of host cell protein expression with high levels of β-galactosidase, infection with SFV(PD)-LacZ permitted a substantial amount of host protein synthesis as compared to uninfected neurons (Fig. 1C). These metabolic labeling experiments were conducted on cell populations; it is thus possible that infection with SFV(PD) had inhibited host protein synthesis in some cells more than in other cells, rather than equally in all cells. In any case, our data indicate that SFV(PD) has a substantially reduced impact on the endogenous protein synthesis of cell lines and neuronal cells.

Transgene Expression Levels and Survival of Infected Cells

SFV vectors with the single point mutations S259P, R649D, R650D, and L713P in *nsP2* expressed slightly increased levels of recombinant protein when compared to the original SFV vector (data not shown). However, when we combined the S259P and R650D mutations into SFV(PD), we found a synergistic effect that resulted in a substantial increase in transgene expression. Compared to SFV-GFP, the onset of transgene expression obtained with SFV(PD)-GFP at up to 1 day post-infection was sometimes delayed (for example, Fig. 2C), but the maximal GFP fluorescence levels achieved at 2–3 days post-infection in BHK cells (Figs. 2A, 2B) and rat E18 cortical neurons (Fig. 2C) were ~ 6- and ~ 2-fold higher, respectively. A similar effect was found for the LacZ reporter gene in BHK cells, where SFV(PD)-LacZ caused a 6-fold larger expression than SFV-LacZ (300 ± 2 vs. 50 ± 1 ng β-galactosidase/μl cell lysate, respectively) (Fig. 2D). These results show that the decreased impact of SFV(PD) on host cell protein synthesis (see earlier) is paralleled by increased transgene expression.

SFV and SFV(PD) exhibited substantial differences in their effect on host cell survival. Microscopic examination of BHK and HEK 293 cells revealed that at 37 °C SFV(PD)-GFP caused less cytopathic effect (CPE, rounding up and detachment of cells) than SFV-GFP (Fig. 3A, right, and data not shown). Whereas most SFV-GFP-infected cells showed CPE within 48 hours, the majority of SFV(PD)-GFP-infected cells appeared intact for at least 5 days. In agreement with this observation, the time course of GFP as well as β-galactosidase expression in BHK cells infected with SFV(PD) was longer than for cells infected with SFV (10 vs. ~ 6 days, respectively; Figs. 2A, 2D). A similar prolonged expression was found in rat E18 cortical neurons (Fig. 2C). Only when SFV(PD) was used at higher concentration (multiplicity of infection (MOI) > 5 and > 1 for BHK cells and P4–5 hippocampal neurons, respectively), cytotoxicity was observed (data not shown). In both BHK cells and neurons, the decrease in GFP fluorescence found after 3 days for wild-type SFV and 7 days for SFV(PD) was mostly due to the lysis of infected cells as well as, presumably, the possible antiviral agents, such as interferon, induced under conditions in which host cell protein synthesis is not inhibited. In any case, our results demonstrate that survival of infected BHK and HEK 293 cells as well as primary neurons is longer for SFV(PD) than for wild-type SFV. This extended cellular survival presumably relates to SFV(PD) still permitting host cell protein synthesis (compare with earlier discussion).

The L713P mutation in nsP2 has earlier been described to permit persistent SFV RNA replication in BHK cells [9]. We thus introduced it into SFV(PD) to generate the triple mutant SFV(PD713P). Interestingly, this vector had a temperature-sensitive phenotype with very low transgene expression at 37 °C (Figs. 2B and 3A, right). However, when

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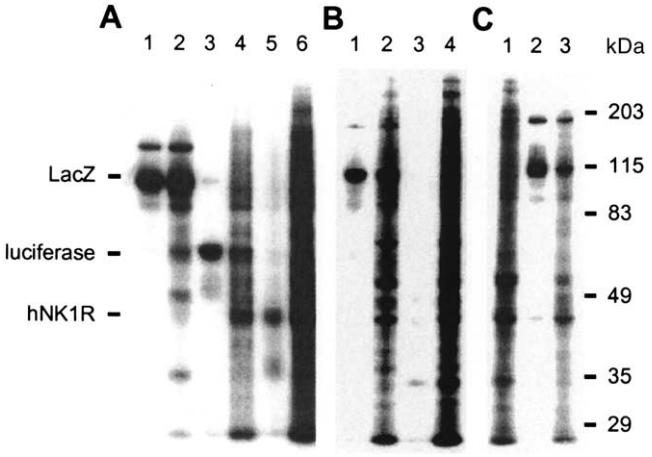

FIG. 1. Metabolic labeling of BHK and HEK 293 cells as well as rat hippocampal neurons infected with conventional SFV and SFV(PD). Expression was verified by 10% SDS-PAGE. Autoradiograms of cells incubated at 37 °C and labeled with [^{35}S]methionine at 16 hours post-infection. (A) BHK cells infected with SFV-LacZ (lane 1), SFV(PD)-LacZ (lane 2), SFV-luciferase (lane 3), SFV(PD)-luciferase (lane 4), SFV-hNK1R (lane 5), and SFV(PD)-hNK1R (lane 6). (B) HEK 293 cells infected with SFV-LacZ (lane 1), SFV(PD)-LacZ (lane 2), SFV-hNK1R (lane 3), and SFV(PD)-hNK1R (lane 4). (C) Uninfected rat E18 hippocampal neurons (lane 1) and neurons infected with SFV-LacZ (lane 2) and SFV(PD)-LacZ (lane 3). Note the dark "smears" of radioactively labeled host cell proteins in uninfected neurons and cells infected with SFV(PD), which are absent in cells infected with wild-type SFV. The bands for the expressed transgenes are marked on the left and the molecular weights of the marker proteins are indicated on the right.

SFV(PD713P)-GFP-infected BHK cells were cultured at 31 °C, the GFP expression levels obtained at ≥ 3 days post-infection were similar or even higher than those obtained with SFV(PD)-GFP (Figs. 2A and 3A, left). At this temperature, the maximal GFP expression levels obtained with SFV(PD713P) were doubled as compared to the ones yielded with SFV(713P) carrying the single L713P mutation (Fig. 2B). This result shows that SFV(PD713P) provides stronger transgene expression than the original SFV(713P) mutant described by Perri *et al.* [9].

Most importantly, whereas SFV(PD)-mediated GFP expression declined with time, SFV(PD713P) allowed it to proceed at elevated levels for 20 days (that is, the longest period tested; Fig. 2A). In accordance with this finding, SFV(PD713P)-infected cells showed hardly any CPE and could even undergo mitosis and be recultured (data not shown). These results agree with the earlier described persistence of the L713P mutant SFV [9] and suggest that SFV(PD713P)-encoded viral replication continues for an extended period. Considering all these results, SFV(PD713P) allows both high-level and long-term transgene expression.

Temperature Effects

We have demonstrated earlier that the temperature used for culturing SFV-infected cells can substantially affect

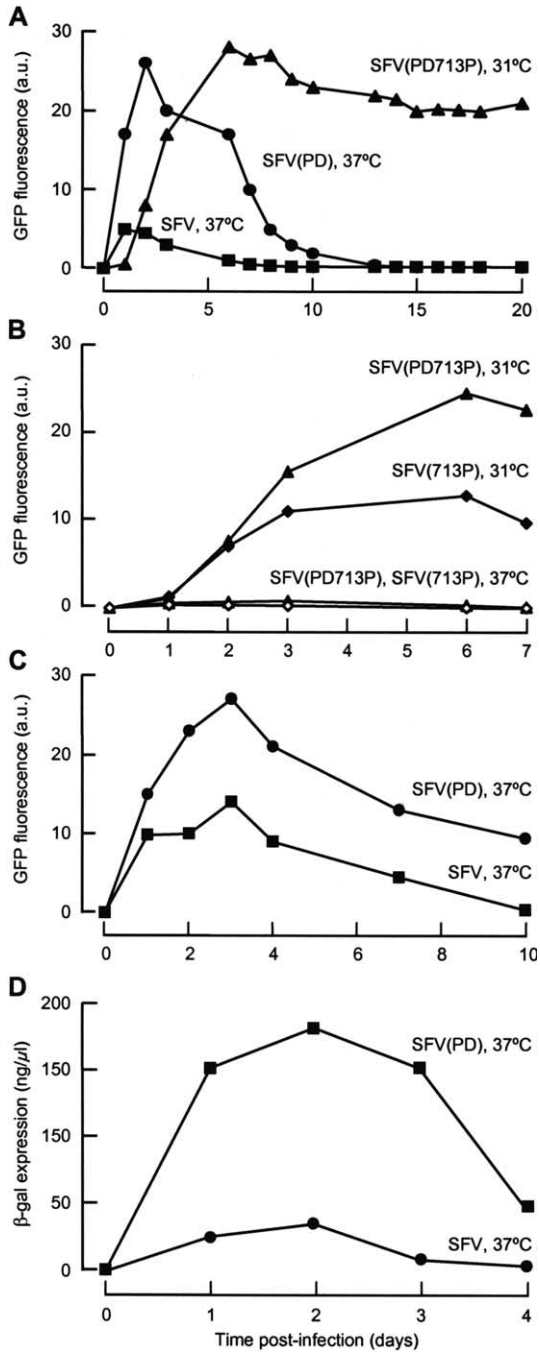

FIG. 2. Time course of transgene expression in BHK cells (A, B, D) and rat E18 cortical neurons (C). Cells were infected with wild-type SFV-GFP (squares), SFV(PD)-GFP (circles), SFV(PD713P)-GFP (triangles), SFV(713P)-GFP (open diamonds), (D) SFV-LacZ (circles) and SFV(PD)-LacZ (squares). Relative fluorescence was measured in living cells at increasing time points post-infection. Cells infected with SFV-GFP and SFV(PD)-GFP were cultured at 37 °C, whereas cells infected with SFV(PD713P)-GFP and SFV(713P)-GFP were cultured at both 37 °C and 31 °C (as indicated). Note that the maximal fluorescence levels for SFV(PD) are similar to the ones for SFV(PD713P) (A), which in turn are higher than for SFV(713P) (B). β-Galactosidase activity was measured in homogenates from parallel cultures at various time points post-infection (D). a.u., Arbitrary units.

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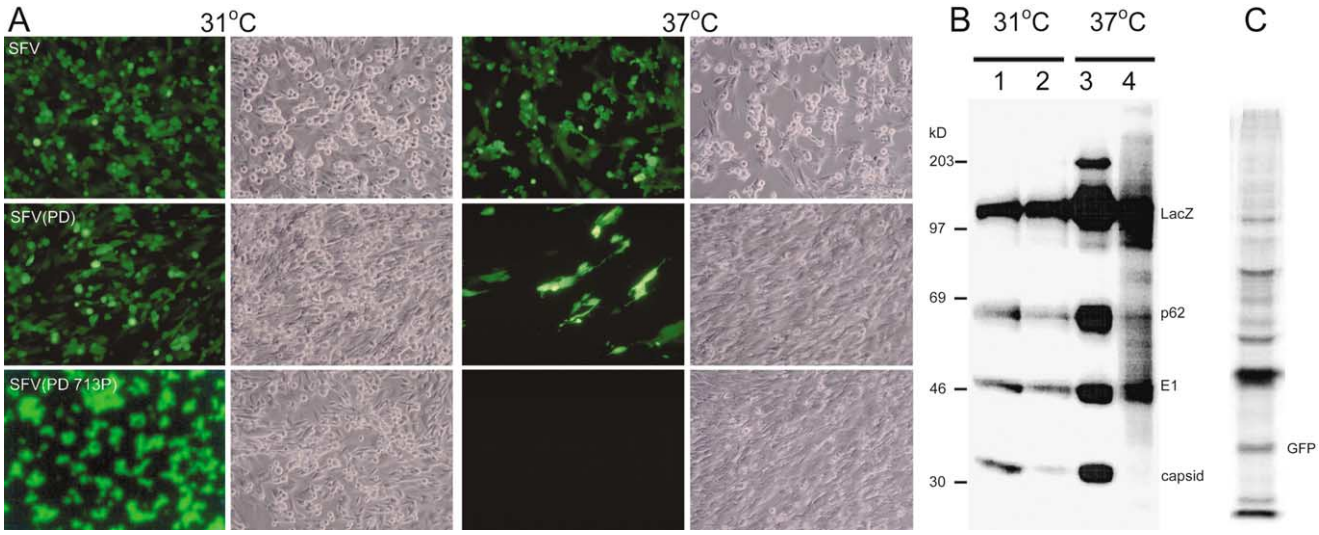

FIG. 3. Temperature effect on GFP expression and morphology (A) as well as protein synthesis (B, C) in BHK cells. (A) Fluorescence and phase contrast micrographs of cells at 3 days after infection with SFV-GFP (top), SFV(PD)-GFP (middle), and SFV(PD713P)-GFP (bottom), and culturing at 31 °C and 37 °C (left and right, respectively). (B) Metabolic labeling of cells co-electroporated with RNA from pSFV-Helper2 and wild-type pSFV3-LacZ (lanes 1 and 3) or pSFV(PD)-LacZ (lanes 2 and 4). Cells were incubated at 31 °C and 37 °C (lanes 1–2 and 3–4, respectively). Expression was verified by 10% SDS-PAGE followed by autoradiography. Note the absence of labeled host cell proteins in lane 2. The bands for the expressed viral proteins (LacZ reporter, p62 precursor for spike proteins E3 and E2, spike protein E1, and capsid protein) and the molecular weights of marker proteins are indicated. (C) Metabolic labeling of BHK cells (31 °C) infected with SFV(PD713P)-GFP for 16 hours and verified by SDS-PAGE and autoradiography.

transgene expression levels [16]. In addition, many mutations in *nsP1–nsP4* of alphaviruses cause temperature-sensitive phenotypes [10–12]. We therefore investigated for SFV(PD) and SFV(PD713P) the effect of altered temperature on virus production, transgene expression, and host cell protein synthesis in BHK cells.

The titers of SFV(PD) replicons obtained at 31 °C were 5- to 10-fold higher than at 37 °C. In contrast, this temperature change had no substantial effect on the production of the SFV4-based replicons. The production of SFV(PD713P) replicons was highly temperature-dependent, again. Extremely low titers resulted at 37 °C ($< 10^3$ infectious particles/ml), whereas higher titers occurred at 31 °C (10^7 infectious particles/ml). Table 1 summarizes the titers obtained for the different viral constructs at 31 °C and 37 °C.

Whereas changing the temperature from 37 °C to 31 °C did not substantially alter the GFP fluorescence levels obtained with conventional SFV, the levels for SFV(PD) at 37 °C were at least 5-fold higher at 31 °C, when they were comparable to the ones for conventional SFV (Fig. 3A): the mean fluorescence at 37 °C was 49,800 and 272,800 arbitrary units (a.u.) for SFV-GFP and SFV(PD)-GFP, respectively. SFV(PD713P)-mediated GFP expression showed an even more pronounced temperature sensitivity, in that no detectable levels occurred at 37 °C (Fig. 3A, right), but at 31 °C they were similar to or higher than the ones for SFV(PD) (Figs. 2A and 3A, left). Table 1 gives an overview of the transgene expression levels obtained at 31 °C versus 37 °C.

The effect of SFV(PD) on host cell protein synthesis strongly depended on the incubation temperature. Similar to cells infected with SFV(PD) (Fig. 1), host cell protein synthesis continued to occur in cells electroporated with SFV(PD) RNA (Fig. 3B, lane 4). However, when SFV(PD) RNA-transfected cells were cultured at 31 °C, host cell

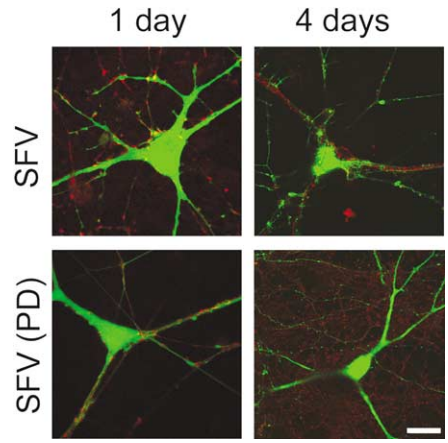

FIG. 4. Infection of dissociated rat hippocampal neurons. Dissociated rat hippocampal neurons prepared from P4–5 rats were cultured for 2 weeks and then infected with wild-type SFV-GFP (top) and SFV(PD)-GFP (bottom). Fluorescence micrographs show the GFP fluorescence (green) and synaptic vesicle cycling (red) of neurons at 1 and 4 days post-infection (left and right, respectively). Note the abnormal dendritic tree and the reduced number of active synapses in neurons infected with wild-type SFV, both very consistent findings at 4 days post-infection. Bar represents 100 μm.

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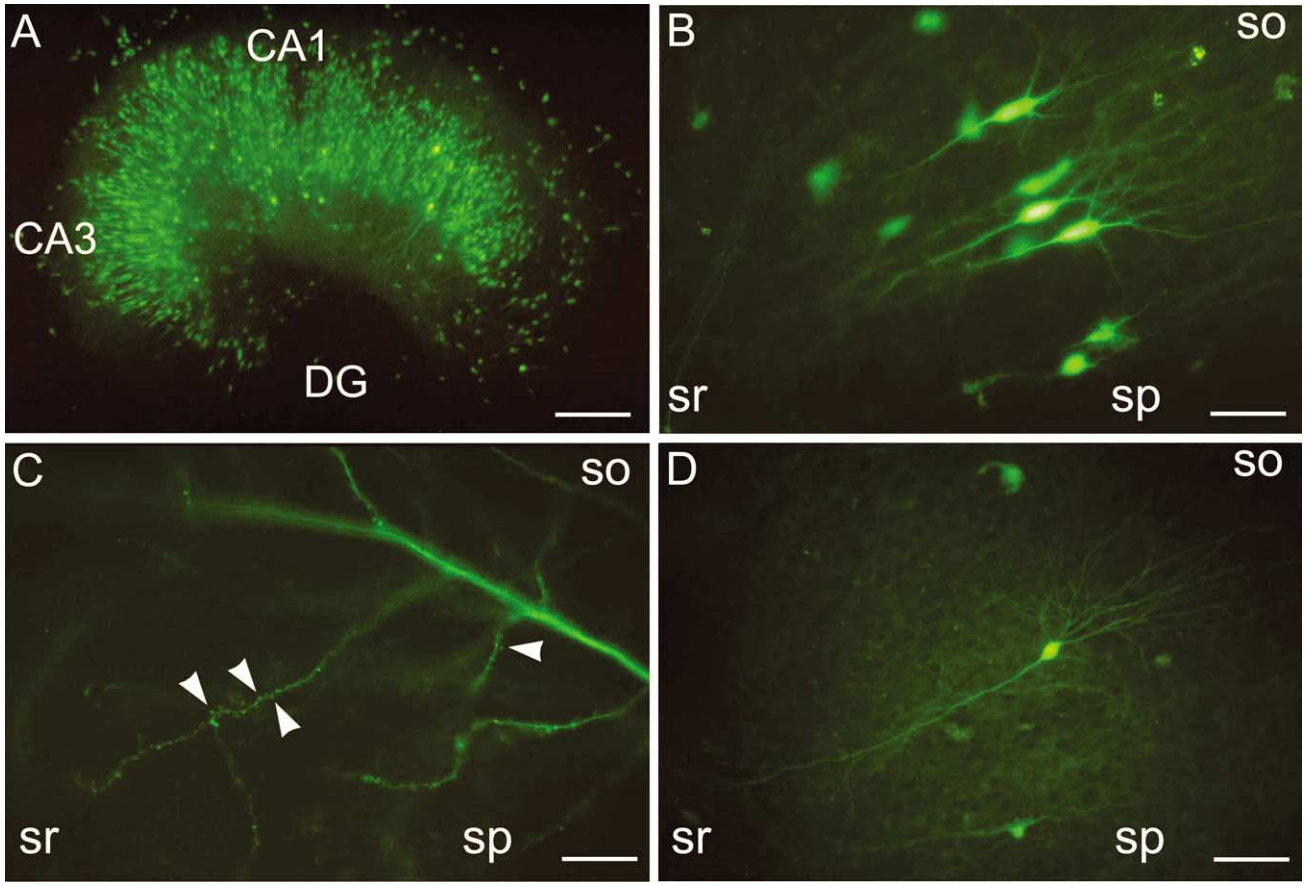

FIG. 5. Infection of cultured rat hippocampal slices. Fluorescence illuminations of slices at 2 and 3 days after injection of SFV(PD)-GFP (A–C) and SFV(PD713P)-GFP (D), respectively, into stratum pyramidale. Upon infection, slices were cultured at 37 °C (A–C) and 31 °C (D). (A) Whole slice; (B, D) CA3 and CA1 pyramidal cells, respectively; (C) apical dendrites in CA1 stratum radiatum with arrowheads pointing to selected GFP^+ spines. DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Bars represent 320 μm (A), 80 μm (B, D), and 25 μm (C).

protein synthesis was efficiently turned off (Fig. 3B, lane 2), as was the case for cells transfected with RNA for SFV (Fig. 3B, lanes 1 and 3), and mainly synthesis of virus-encoded proteins occurred. In agreement with the block of host cell protein synthesis at 31 °C, cells infected with SFV(PD) displayed massive CPE at 31 °C in a manner similar to conventional SFV (Fig. 3A, left). Interestingly, as described earlier, infection with SFV(PD713P) at 31 °C did not cause any CPE but rather permitted continuous cellular survival and transgene expression (compare Fig. 2A). In any case, our data show that SFV(PD) exhibits a less cytopathic phenotype at 37 °C but not 31 °C. Again, Table 1 summarizes the effect of the incubation temperature on host cell protein synthesis and survival after infection with the different viral constructs.

Survival and Synaptic Activity of P4–5 Hippocampal Neurons

To avoid overinfection of neurons (see earlier), we used SFV-GFP and SFV(PD)-GFP at 100 and 1,000 particles/ml, respectively. At these concentrations, very few neurons per dish were infected. They were imaged 1 and 4 days post-infection. Whereas infection with SFV induced abnormal neuronal morphologies in the form of swellings of dendritic processes followed by the complete disorganization of dendrites and CPE (Fig. 4, top right panel), SFV(PD)-infected neurons remained morphologically intact (Fig. 4, bottom right panel). As a measure of neuronal function, we examined presynaptic quantal release by monitoring vesicular uptake of extracellular tracers. In these experiments, evoked quantal release was triggered by electrical stimulation at 0.5 Hz in the presence of anti-synaptotagmin-I antibodies. These antibodies specifically recognize the luminal domain of the synaptic vesicle protein and, upon exocytotic fusion, bind to exposed synaptotagmin-I and are then taken up into the presynaptic terminal by vesicle recycling [17]. Our quantitative analysis of the immunoreactivity in axon terminals at both 1 and 4 days post-infection showed that SFV(PD)-

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Table 1: Comparison of the conventional and novel SFV vectors as a function of incubation temperature

	Titers obtained (particles/ml)		Transgene expression		Host protein synthesis		Induction of CPE	
Vector	31 °C	37 °C	31 °C	37 °C	31 °C	37 °C	31 °C	37 °C
SFV	$\sim 10^9$	$\sim 10^9$	+	+	−	−	+	+
SFV (PD)	$0.5–1 \times 10^8$	$0.5–2 \times 10^7$	+	++	−	+	+	−
SFV (PD713P)	$\sim 10^7$	$<10^3$	+	−	+	n.a.	−	n.d.

The table summarizes the viral titers obtained at 31 °C and 37 °C and schematically summarizes transgene expression, effect on host cell protein synthesis, and induction of cytopathic effects (CPE) of the vectors at both temperatures. Note that the incubation temperature drastically alters the behavior of SFV(PD) and SFV(PD713P). −, No detectable expression; +, relatively high expression; ++, 2- to 5-fold higher expression; n.d., not determined; n.a., not applicable.

infected neurons not only retain a normal morphology but also display normal synapses with detectable levels of evoked synaptic exo- and endocytosis. On the contrary, wild-type SFV-infected cells displayed very few active synapses at 4 days post-infection (Fig. 4, top right panel). Taken together, our data demonstrate that SFV(PD), as compared to SFV, maintains unaltered synaptic physiology and permits substantially longer survival of hippocampal neurons.

Infection of Hippocampal Slices

We have demonstrated earlier high neuronal specificity of SFV-mediated transgene expression by using SFV vectors in rat hippocampal slices [18]. Here, we injected SFV(PD)-GFP at 10- to 1000-fold dilution, corresponding to $\sim 10^5$ to 10^3 infectious particles, into hippocampal slices and then incubated them at 37 °C. GFP fluorescence could be detected at 1 day post-infection and was increased at 2 days, when it allowed the unambiguous identification of infected CA1 and CA3 pyramidal cells (Figs. 5A, 5B). At this and subsequent time points, GFP fluorescence could easily be detected in very distal and thin dendrites of up to 4th order and even in presumptive spines (Fig. 5C). GFP^+ neurons appeared morphologically intact for at least 4 days post-infection. Upon application of 200 SFV(PD)-GFP particles, an average of 105 cells in the injected area per slice expressed GFP fluorescence at 1–2 days post-infection; $\sim$ 87% of them were pyramidal cells and $\sim$ 5% interneurons [19]. Overall, these findings are similar to the results earlier obtained with SFV-GFP [18] and show that SFV(PD) also efficiently transduces neurons in rat hippocampal slices.

We also applied SFV(PD713P)-GFP to hippocampal slices. Because the titers obtained for SFV(PD713P)-GFP were substantially lower than for wild-type SFV and SFV(PD), we injected undiluted SFV(PD713P)-GFP and a 10-fold dilution. Upon incubation at 31 °C, only single GFP^+ pyramidal cells were found for the 10-fold dilution (n = 12 slices). The undiluted virus, by contrast, resulted in an average of 20 ± 5 GFP^+ cells per slice (n = 5 slices). Similar to BHK cells, no GFP expression was detected when the slices were cultured at 37 °C. Figure 5D shows a SFV(PD713P)-GFP-infected CA1 pyramidal cell at 3 days post-infection. As a result of the high expression levels obtained at 31 °C, GFP fluorescence could be detected in extremely distant and thin dendrites. Again, these data are compatible with the efficient transduction of neurons in tissue slices that we earlier obtained with wild-type SFV and SFV(PD) [18,19].

Discussion

We have engineered two novel SFV vectors, SFV(PD) and SFV(PD713P), that generate infectious recombinant particles and, upon infection of rodent and human cell lines as well as neurons, bring about 2- to 6-fold elevated levels of expressed recombinant protein, as compared to the wild-type SFV vector. A similar increase in transgene expression has been earlier described in SIN for the S726P mutation in *nsP2* (P726S) [7]. In addition, both SFV(PD) and SFV(PD713P) led to substantially prolonged host cell survival and transgene expression, with SFV(PD713P) causing persistent GFP expression. The decrease of cytotoxicity obviously results from the ability of these replicons—in contrast to conventional SFV—to permit the synthesis of host cell proteins, which are required for infected cells to survive. Such a reduced inhibition of endogenous protein synthesis also underlies the persistent infection obtained in BHK cells with SIN carrying the P726S mutation in *nsP2* [7]. Because uninfected cells synthesize more total protein than alphavirus-infected cells [4], we assume that these SFV and SIN mutants cause elevated transgene expression by providing infected cells with a higher ability for total protein synthesis.

Because nsP2 is the protease responsible for cleaving the nsP1–nsP4 polyprotein, the L713P mutation might result in altered proteolytic activity. However, no such alteration was found for the corresponding mutation in *nsP2* from SIN [7]. In SFV-infected cells, about half of the nsP2, which also contains NTPase activity [20], is transported to the nucleus [21]. A point mutation (R649D) in the nuclear localization signal (P^{648}RRV) of nsP2 made it cytoplasmic [22] and, when incorporated into SFV particles, reduced the damage of host cell DNA synthesis and

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the pathogenicity in mice [13]. Because the R650D mutation in SFV(PD) also lies within the nuclear localization signal, we suggest that the nsP2 of SFV(PD) remains cytosolic in host cells. In support of this hypothesis, our preliminary data indicate that this is the case, at least for BHK cells, in which no nsP2 was detected in the nucleus, but was retained in the cytoplasm based on immunofluorescence microscopy using nsP antibodies (M. J. Fend and M. U. Ehrengruber, unpublished results).

Importantly, when SFV(PD) was used at higher MOI, it was cytotoxic to both BHK cells and hippocampal neurons in a manner similar to SFV. The reason may be that the extreme amounts of viral RNA molecules generated by multiple viral genomes in a given cell efficiently compete with endogenous RNA molecules for the cellular translation machinery. In addition, it has been shown that double-strand RNA molecules generated during alphaviral replication induce interferon, which can upregulate cytotoxic protein kinase-RNA and 2′,5′-oligoadenylate [23]. However, our preliminary DNA microarray analysis using HEK 293 cells did not identify any interferon gene to be induced by SFV(PD) (U. Certa and K. Lundstrom, unpublished results). In any case, experiments aiming at 100% infection levels with SFV(PD) should be designed carefully to prevent overinfection and, thus, cytotoxicity. It is therefore highly advisable to conduct titration experiments for various host cells to find out the limits for cytotoxicity. For instance, in BHK cells and neurons, MOI values < 5 and < 1, respectively, are suggested.

It is interesting that SFV(PD) is less cytotoxic at 37 °C but not at 31 °C. The two mutations in nsP2 might affect viral RNA replication at 37 °C rather than at 31 °C. In support of this idea, the titer of SFV(PD) stocks generated at 37 °C was substantially lower than at 31 °C. Interestingly, SFV(PD713) behaved quite differently than SFV(PD). It was strongly temperature sensitive and had to be packaged at the permissive temperature (31 °C), but it was not cytotoxic at this temperature.

In summary, our novel, less cytopathic SFV mutants will allow application of SFV vectors under conditions at which the cell functions are not compromised by endogenous gene inhibition and early onset of apoptosis. In addition to general overexpression of recombinant proteins, the SFV technology can now be used more efficiently to study expression kinetics and signal transduction events. Additionally, the absence of the general inhibition of endogenous gene expression should further increase the application range of the SFV mutants. For instance, antisense and RNA interference approaches should be especially attractive because of the extreme cytoplasmic RNA amplification from the SFV replicon. Our preliminary results demonstrated that co-infection with SFV(PD) carrying sense and antisense target sequences efficiently and specifically reduced the target gene expression in various host cell lines (U. Certa and K. Lundstrom, unpublished results). In the area of gene therapy, SFV(PD) has two advantages over the SFV4-based vector: (1) the higher expression levels results in production of more therapeutic protein, and (2), the prolonged survival of host cells allows for improved treatment efficacy. As compared to the earlier described less cytopathic and noncytopathic SIN vectors [7,8,24], the advantages of SFV(PD) and SFV(PD713P) are the wider host cell range (cell lines and neurons) and, at least for SFV(PD), the higher replicon titers obtained.

MATERIALS AND METHODS

Cell and tissue cultures. BHK, Chinese hamster ovary, and HEK 293 cells were cultured in a 1:1 mixture of Dulbecco's modified F-12 medium (Invitrogen) and Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 4 mM glutamate and 10% FCS. Primary embryonic day 18 (E18) rat hippocampal and cortical neurons were cultured in Neurobasal medium (Invitrogen) on 24-well plates, while postnatal day 4–5 (P4–5) rat hippocampal neurons were cultured on glass coverslips in 35-mm petri dishes as described [17]. Briefly, the CA1 and CA3 hippocampal regions were removed from 3- to 5-day-old rats, and the neurons recovered by enzymatic digestion (trypsin type XI, 10 mg/ml, plus DNase I type IV, 0.5 mg/ml) and mechanical dissociation. The cells were cultured in minimal essential medium containing 0.6% (wt/vol) glucose, 1 mM glutamine, 2.4 g/L $NaHCO_3$, 100 mg/ml bovine transferrin, 25 mg/ml insulin, 5–10% FCS, and plated at a density of 50,000 cells per 35-mm plastic petri dish (Falcon) coated with polyornithine and Matrigel (Collaborative Research). The cultures were maintained at 37 °C in 95% air, 5% CO_2 in a humidified incubator, and the medium replaced every 3–4 days. From the second day in culture, the culture medium was supplemented with 5 μM cytosine-D-arabinofuranoside. Neurons were used for synaptic uptake experiments at 10–14 days after plating. Hippocampal slices from postnatal day 6 rats were cultured in the roller-tube configuration [25].

Site-directed mutagenesis. Amino acid substitutions in the protein encoded by the SFV *nsP2* gene were carried out by PCR technology. The nsP2–nsP4 region was subcloned as a *Sac*I-*Xba*I fragment from pSFV2gen [26] into pGEM7Zf(+) (Promega). pGEM7Zf(+)-nsP2-4 was then used as a template for PCR reactions with primers designed to substitute the desired nucleotides in the *nsP2* gene. The mutations were confirmed by sequencing, and the entire *Sac*I-*Xba*I fragment reintroduced into pSFV2gen.

Generation of recombinant SFV. RNA from *in vitro*-transcribed pSFV3 encoding *Escherichia coli* β-galactosidase (LacZ) [1], pSFV1 encoding firefly luciferase [27] and human neurokinin 1 receptor (hNK1R) [28], pSFV2gen encoding enhanced green fluorescent protein (GFP) [18], pSFV(PD)-LacZ, pSFV(PD)-luciferase, pSFV(PD)-GFP, pSFV(PD)-hNK1R, pSFV(713P)-GFP, and pSFV(PD713P)-GFP was co-electroporated with RNA from pSFV-Helper2 [29] into BHK cells. Recombinant SFV particles were harvested after 24 hours and activated with α-chymotrypsin, and test infections with serial dilutions of virus expressing GFP and LacZ were carried out for titer determinations as described [30].

Infection and analysis of gene expression. Cell lines and primary neurons were infected by adding undiluted (cell lines) or diluted (neurons) virus to the culture medium. Organotypic hippocampal slices (average age: 15 ± 6 days in culture, mean ± SD, 7 batches; 2–14 sister cultures per batch) were infected and analyzed as described [18,31]. Upon infection, cells and slices were incubated at either 31 °C or 37 °C (as indicated).

Cells were metabolically labeled at 16 hours post-infection with [^{35}S]methionine (Perkin Elmer, Zurich, Switzerland), and protein expression verified by 10% SDS-PAGE and subsequent autoradiography. LacZ expression was visualized by X-gal staining, and enzymatic activity measured as described [1]. GFP fluorescence was monitored in living cells with a Zeiss Axiovert 100 microscope and levels quantified on 24- and 96-well plates by using a Fluoroscan Ascent fluorometer (Labsystems, Helsinki, Finland) with excitation at 480 nm and emission at 538 nm. Synaptic

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activity in dissociated hippocampal neurons was analyzed as described [17].

Analysis of synaptic vesicle cycling and immunocytochemistry. Polyclonal sera used in these studies were obtained from rabbits and goats immunized with a synthetic peptide (NH_2-MVSASHPEALAAPVT-TVATLVPHC-COOH) corresponding to the intravesicular N-terminal portion of synaptotagmin-I [17,32,33]. These antibodies were affinity-purified as earlier described [17]. For the present experiments, anti-synaptotagmin antibodies were added to the culture medium (supplemented with 25 μM APV (2-amino-5-phosphonovalerate) to block NMDA receptor-mediated currents) at a final dilution of 1:100-1:200, and the hippocampal cell cultures were field-stimulated with platinum wires at 0.5 Hz for 10 minutes (at 37 °C and 95% air, 5% CO_2). At the end of this 10-minute stimulation, cultures were washed extensively with oxygenated Tyrode solution (37 °C) containing 119 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 30 mM glucose, 25 μM APV, and 25 mM HEPES, pH 7.4, and then fixed with 4% paraformaldehyde. Fixed cells were permeabilized in blocking solution (0.4% (wt/vol) BSA, and 0.4% (wt/vol) saponin in 120 mM phosphate buffer), incubated with primary antibodies (monoclonal antibodies against the cytosolic domain of synaptotagmin-I) dissolved in blocking solution (2 hours, room temperature), and, after an extensive wash with blocking solution, incubated at room temperature with species-specific fluorochrome-conjugated secondary antibodies (1 hour, room temperature). Fluorescence images were captured with a one-photon confocal microscope (Zeiss). Quantification of the number of synapses and of the vesicular uptake of anti-synaptotagmin-I antibodies was done using a computer program written in house [32,33]. Synapses were first identified based on intensity and shape parameters, and then fluorescence intensities from internalized antibodies were measured on a different excitation/emission channel from the same areas. Intensity values were determined near the center of individual boutons and used for analysis if they exceeded the SD of the background fluorescence by > 3 times.

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REFERENCES

1. Liljeström, P., and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Bio/Technology* **9:** 1356–1361.
2. Xiong, C., *et al.* (1989). Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* **243:** 1188–1191.
3. Schlesinger, S. (2000). Alphavirus expression vectors. *Adv. Virus Res.* **55:** 565–577.
4. Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.* **58:** 491–562.
5. Peränen, J., Takkinen, K., Kalkkinen, N., and Kääriäinen, L. (1988). Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J. Gen. Virol.* **69:** 2165–2178.
6. LaStarza, M. W., Lemm, J. A., and Rice, C. M. (1994). Genetic analysis of the *nsP3* region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. *J. Virol.* **68:** 5781–5791.
7. Dryga, S. A., Dryga, O. A., and Schlesinger, S. (1997). Identification of mutations in a Sindbis virus variant able to establish persistent infection in BHK cells: the importance of a mutation in the nsP2 gene. *Virology* **228:** 74–83.
8. Agapov, E. V., *et al.* (1998). Non-cytopathogenic Sindbis RNA vectors for heterologous gene expression. *Proc. Natl. Acad. Sci. USA* **95:** 12989–12994.
9. Perri, S., *et al.* (2000). Replicon vectors derived from Sindbis virus and Semliki Forest virus that establish persistent replication in host cells. *J. Virol.* **74:** 9802–9807.
10. Hahn, Y. S., Strauss, E. G., and Strauss, J. H. (1989). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. *J. Virol.* **63:** 3142–3150.
11. Hahn, Y. S., Grakoui, A., Rice, C. M., Strauss, E. G., and Strauss, J. H. (1989). Mapping of RNA-temperature-sensitive mutants of Sindbis virus: complementation group F mutants have lesions in nsP4. *J. Virol.* **63:** 1194–1202.
12. Boorsma, M., *et al.* (2000). A temperature-regulated replicon-based DNA expression system. *Nat. Biotechnol.* **18:** 429–432.
13. Rikkonen, M. (1996). Functional significance of the nuclear-targeting and NTP-binding motifs of Semliki Forest virus nonstructural protein nsP2. *Virology* **218:** 352–361.
14. Fazakerley, J. K., Boyd, A., Mikkola, M. L., and Kääriäinen, L. (2002). A single amino acid change in the nuclear localization sequence of the nsP2 protein affects the neurovirulence of Semliki Forest virus. *J. Virol.* **76:** 392–396.
15. Lundstrom, K., Rotmann, D., Hermann, D., Schneider, E. M., and Ehrengruber, M. U. (2001). Novel mutant Semliki Forest virus vectors: gene expression and localization studies in neuronal cells. *Histochem. Cell Biol.* **115:** 83–91.
16. Schlaeger, E.-J., and Lundstrom, K. (1998). Effect of temperature on recombinant protein expression in Semliki Forest virus-infected mammalian cell lines growing in serum-free suspension cultures. *Cytotechnology* **28:** 205–211.
17. Malgaroli, A., *et al.* (1995). Presynaptic component of long-term potentiation visualized at individual hippocampal synapses. *Nature* **268:** 1624–1628.
18. Ehrengruber, M. U., *et al.* (1999). Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc. Natl. Acad. Sci. USA* **96:** 7041–7046.
19. Ehrengruber, M. U., *et al.* (2001). Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Mol. Cell. Neurosci.* **17:** 855–871.
20. Rikkonen, M., Peränen, J., and Kääriäinen, L. (1994). ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J. Virol.* **68:** 5804–5810.
21. Peränen, J., Rikkonen, M., Liljeström, P., and Kääriäinen, L. (1990). Nuclear localization of Semliki Forest virus-specific nonstructural protein nsP2. *J. Virol.* **64:** 1888–1896.
22. Rikkonen, M., Peränen, J., and Kääriäinen, L. (1992). Nuclear and nucleolar targeting signals of Semliki Forest virus nonstructural protein nsP2. *Virology* **189:** 462–473.
23. Saito, S. (1989). Possible involvement of virus-induced protein kinase in the antiviral state induced with interferon-γ against Sindbis virus. *J. Interferon Res.* **9:** 23–34.
24. Frolov, I., *et al.* (1999). Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. *J. Virol.* **73:** 3854–3865.
25. Gähwiler, B. H. (1981). Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Methods* **4:** 329–342.
26. Malherbe, P., *et al.* (1999). Cloning and functional expression of alternative spliced variants of the human metabotropic glutamate receptor 8. *Mol. Brain Res.* **67:** 201–210.
27. Lundstrom, K., *et al.* (2001). Improved Semliki Forest virus vectors for receptor research and gene therapy. *J. Recept. Signal Transduct. Res.* **21:** 55–70.
28. Lundstrom, K., *et al.* (1994). High-level expression of the human neurokinin-1 receptor in mammalian cell lines using the Semliki Forest virus expression system. *Eur. J. Biochem.* **224:** 917–921.
29. Berglund, P., *et al.* (1993). Semliki Forest virus expression system: production of conditionally infectious recombinant particles. *Bio/Technology* **11:** 916–920.
30. Ehrengruber, M. U., and Lundstrom, K. (2000). Alphavirus-mediated gene transfer into neurons. In *Current Protocols in Neuroscience* (J. N. Crawley, *et al.*, Eds.), pp. 4.22.1–4.22.23. John Wiley & Sons, New York.
31. Ehrengruber, M. U., Ehler, E., Billeter, M. A., and Naim, H. Y. (2002). Measles virus spreads in rat hippocampal neurons by cell-to-cell contact and in a polarized fashion. *J. Virol.* **76:** 5720–5728.
32. Böse, C. M., *et al.* (2000). Agrin controls synaptic differentiation in hippocampal neurons. *J. Neurosci.* **20:** 9086–9095.
33. Ingrassia, R., Trabucchi, C., Bergamaschi, A., and Malgaroli, A. (1999). Monitoring synaptic exocytosis and endocytosis by optical techniques. In *Imaging Living Cells* (R. Rizzuto and C. Fasolato, Eds.), pp. 231–261. Springer, Heidelberg.

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