Semliki Forest virus-derived virus-like particles: characterization of their production and transduction pathways

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A procedure for the mobilization of Semliki Forest virus (SFV)-derived replicons using virus-like particles (VLPs) has been recently proposed. VLPs were obtained from 293T cells co-expressing the vesicular stomatitis virus glycoprotein (VSV-G) and a modified SFV replicon. Advantages of SFV VLPs include improved safety with a lack of sequence homology between components and reducing the risk of recombination events that could lead to the formation of autonomous particles. Characterization of SFV VLPs reveals a discrepancy in their ability to infect cells reported to be permissive. Furthermore, it was noted that not all viral envelopes were able to promote VLP release equally from transfected cells. These observations encouraged the examination of the molecular mechanisms supporting the different steps of VLP assembly and transduction. The use of a VSV-G related pathway for VLP entry into target cells was demonstrated; it was also observed that an internal ribosome entry site may not be adapted to control transgene expression in all cells. Finally, the need for a membrane-binding domain to obtain a fully active SFV replication complex and VLP formation was documented.

INTRODUCTION

The use of alphavirus-derived vectors for cancer gene therapy has been extensively explored in recent years (Berglund et al., 1998; Leitner et al., 2000, 2003; Lundstrom, 2001; Smyth et al., 2005; Vasilakis et al., 2003). In vivo vector delivery is the preferred mode of administration and therefore represents a key factor in determining the specificity as well as the efficacy of the treatment. Here, the use of recombinant particles might confer advantages over the use of naked DNA or RNA (Larin et al., 2004). Virus-driven tumoral cytotoxicity relies on two non-exclusive techniques, cytoytic replicative viruses or suicidal recombinant viral vectors, and several genera of viruses have been used to target cancer cells (Everts & Poel, 2005; Fielding, 2005; Rheme et al., 2005).

Recently, it has been suggested that alphaviruses harbour some replication specificity in tumour cells (Tseng et al., 2004a, b). However, one case of Semliki Forest virus (SFV)-induced encephalitis has been described in humans, tempering the use of replication competent SFV as a therapeutic agent (Willems et al., 1979). Therefore, defective SFV vectors are a pertinent alternative. SFV vectors additionally offer the opportunity to deliver locally high levels of bioactive proteins (Ehrengruber, 2002). Production of recombinant SFV particles is possible through the transcomplementation of SFV replicons with viral structural proteins (Lundstrom, 2002; Lundstrom et al., 2001). Important modifications have been introduced to eliminate recombination events that lead to the production of replication competent particles (Ehrengruber, 2002; Smerdou & Liljestrom, 1999). Alternatively, we have previously shown that it is possible to mobilize SFV replicons by making VLPs (Dorange et al., 2004). These particles consist of a membrane anchored vesicular stomatitis virus glycoprotein (VSV-G) that forms a lipoprotein envelope encapsulating the SFV replicon. Rolls and collaborators have shown that such VLPs are able to package genomic as well as subgenomic RNAs (Rolls et al., 1994, 1996). To avoid packaging subgenomic RNAs, we have introduced several disabling mutations into the internal 26S promoter (Dorange et al., 2004). The modified replicon shows defective subgenomic RNA synthesis leading to exclusive packaging of the genomic RNA. In order to direct transgene expression at a detectable level, this modification additionally requires a translation initiator, in this case, a viral encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) element.

Aiming at an efficient vector delivery, it is important to control every step leading to transgene expression. Using viral vectors, the first limiting step in transduction is cell entry. Most viruses enter target cells using a receptor-dependent pathway. For enveloped virus, entry is via either an endocytic route or by direct membrane fusion (Sandrin et al., 2003). Experimentally, the two processes are
distinguished by the low pH requirement of the endocytic route. The SFV VLPs used in this study are coated with the VSV-G envelope protein, so we focused on the endocytic pathways acknowledged to be the route of entry for the parental VSV particles. An advantage of VSV-G is its ability to confer a wide tropism to the VLPs. However, we noted that certain cells were non-permissive to the modified SFV based vectors (Doranne et al., 2004). Surprisingly, HeLa cells, which are known to express SFV replicons and are susceptible to transduction by VSV-G pseudotyped retroviral vectors, proved to be non-permissive to the VLPs. This suggested that other factors influence replicon based expression.

We also examined the subcellular sites at which the VLPs are budding. Both the SFV nsP1 and the VSV-G are palmitoylated (Ahola et al., 2000; Bijlmakers & Marsh, 2003). The precise role of VSV-G palmitoylation is unclear since, being a membrane-spanning protein, VSV-G is constantly expressed at the cell surface. We note that palmitoylation acts to localize proteins within specialized membrane domains (Bijlmakers & Marsh, 2003). Among these, the lipid rafts have a central role in virus biology, participating in viral entry as well as viral release (Bhattacharya et al., 2004; Holm et al., 2003; Suomalainen, 2002). Moreover, nsP1 palmitoylation has been linked to the formation of filopodia at the cell surface of expressing cells (Ahola et al., 2000). It has also been shown that the nsP1 participates in the transient positioning of the SFV replication complex at the inner side of the cell membrane and the endosomal compartment (Salonen et al., 2003). However, SFV replicons have not been shown to spontaneously bud out of the expressing cell. The palmitoylated murine leukaemia retroviral envelope is able to trigger VLP production when expressed from an SFV replicon (Lebedeva et al., 1997; Li et al., 2002). It was therefore important to evaluate the contribution of palmitoylation to VLP formation. For this purpose, we constructed a series of VSV-G and nsP1 expression vectors harbouring mutations of the palmitoylated cysteines. Using these constructs, we determined the cellular localization of the different components forming the VLPs, and we describe their importance for VLP function.

METHODS

**Cell lines and culture conditions.** Human embryonic kidney 293T cells and HeLa cells were cultured in Dulbecco’s modified essential medium (DMEM; Invitrogen) supplemented with 10% heat inactivated fetal calf serum (FCS; Biowest), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). Baby hamster kidney cells (BHK-21) were cultured in Glasgow MEM BHK-21 (Invitrogen) supplemented with 5% heat inactivated FCS, 8% tryptose phosphate broth solution (Sigma), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}).

**Vectors.** The 26Sm2 SFV vector was generated as previously described (Doranne et al., 2004). Mutation of the nsP1 palmitoylation site (Amp 1251–1260 from nsP1 AUG) was performed by PCR using the following primers: sense 5’-GGTACACTTCTCTTGTGGGAT-3’ and antisense 5’-ATGCCACAGCAAATACTGAGC-3’. The PCR product was purified and then used as template in nested-PCR with the following flanking primers: sense 5’-TAGCATGCGGATGTGTG-3’ and antisense 5’-CCGGTATATGTTATTAT-3’. The PCR product was cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). 26Sm2 was digested with EcoRI/NotI and pCR2.1 was digested with EcoRV and SacI, and the fragment ligated to a fragment obtained from pCR2.1 containing the SFV nsP1, also digested by EcoRV and SacI. This ligation led to the formation of the nsP1 palm-SFV vector.

pMDG encodes the VSV-G cDNA under the control of the cytomegalovirus immediate-early (CMV-IE) promoter. Mutation of the palmitoylation site was performed by PCR using a mismatch (in bold)-containing sense primer 5’-TCCATCTTGGCATTTAAAT-3’ and antisense primer 5’-ATTTAATGCAGATGGA-3’. The PCR product was reamplified using the following primers: sense 5’-AGCGGTCTTCTCCAATCTC-3’ and antisense 5’-CAACAGACACACT-AAGG-3’. The final product was cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). An A gel–Msd fragment was then cloned into pMDG, cut A gel/Msd leading to pMDG-palm(–).

SP6 265m2 was generated as follows: first the mutated 26S promoter sequence was cloned between BglII and BamHI into pIRE52-EGFP (enhanced green fluorescent protein; Clontech). An EcoRI retroviral sequence (RS), from Moloney murine leukaemia virus, was cloned between the 26S sequence and the IRES at BamHI. A 268-RS-IRES-EGFP fragment was excised by BgIII/Hpol, and cloned into pSV1 (Invitrogen) at the BamHI/Smal leading to SP6 265m2 SFV. The nsP1 palm(–) sequence was also introduced into this vector. All modified vectors were sequenced.

**Transfection and production of infectious VLPs.** 293T cells were plated at 8 × 10^5 cells per well in six-well plates and grown overnight at 37 °C. The culture medium was changed 3 h before transfection. Transfections were carried out using a calcium phosphate transfection kit (Invitrogen) according to the manufacturer’s instructions. Sequential transfections were performed as follow: 5 μg pMDG was transfected on day 1 and 5 μg 265m2 SFV vector was transfected on day 2. The second transfection medium was left for 12 h and then removed and replaced by fresh medium to allow release of VLPs. Supernatants were collected and filtered 12 h later. The retroviral vector, pBullet-EGFP, pMNag-pol and pCMV-Ampho, was used as a positive control.

For localization studies, 293T cells were plated at 1 × 10^6 cells per well on culture slides (Falcon). Cells were co-transfected with calcium phosphate as described, using 2-5 μg pMDG or pMDG-palm(–) and 2-5 μg nsP1 SFV or nsP1 palm(–). After 24 h, immunofluorescence staining was performed. The pBullet-EGFP plasmid was used as an internal transfection control.

RNAs from SP6 nsP1 palm(–) 265m2 SFV vector were transcribed in vitro from linearized vectors using SP6 RNA polymerase (Invitrogen). RNAs were transfected into 4 × 10^5 BHK-21 cells by electroporation. Supernatants were collected 16 h later and filtered for 293T cell transduction.

**Target cell transduction.** BHK-21 target cells were plated at 2 × 10^5 cells per well in 24-well plates and grown overnight. Supernatants from transfected 293T cells were collected, filtered through a 0.4-5 μm Millex HA filter (Millipore) and used to transduce BHK-21 cells in the presence of a final volume of 5 μg polybrene (Sigma) ml^{-1}.

All drugs were purchased from Sigma and the anti-VSV-G antibodies were kindly provided by Dr Douglas Lyles (Wake Forest University School of Medicine, North Carolina, USA) (Lefrancois & Lyles, 1982).
Drugs were evaluated in duplicate in three independent experiments and were used at the following concentrations: chloroquine (50, 100 and 150 μM), brefeldin A (1:25, 2:5 and 5 μg ml⁻¹) and bafilomycin A1 (50 and 100 μM dissolved in DMSO). BHK-21 cells were incubated for 1 h prior to transduction in the presence of individual compounds. The culture medium was then removed and BHK-21 cells were transduced with viral supernatants supplemented with the drugs. Infected cells were examined for EGFP expression with an IX50 Olympus microscope equipped with a fluorescent illuminator.

**Flow cytometry.** After overnight incubation (see above), transduced or transfected cells were detached with trypsin-EDTA (Invitrogen), centrifuged and resuspended in PBS for analysis using a FACScalibur flow cytometer (Beckton Dickinson).

**Immunofluorescence and confocal microscopy.** Indirect immunofluorescence was carried out for transfected 293T monolayer cells on coverslips. Cells were washed twice in PBS and fixed in glacial acetone for 10 min. The cells were then incubated, for 1 h at room temperature, with the primary antibodies mouse anti-VSV-G (1:400) and rabbit anti-nsP1 (1:1000) (kindly provided by Dr Leevi Kääriäinen, Institute of Biotechnology, Helsinki, Finland) in 0.5 % PBS/BSA. Three washes with 0.5 % PBS/BSA were performed and cells were incubated with the secondary fluorescent antibodies anti-mouse 488 (Alexa) (1 : 1000) and anti-rabbit 594 (Alexa) (1 : 1000), respectively. After three washes, slides were fixed and mounted with Immuno-Fluor (MP Biomedicals).

The samples were analysed using a confocal microscope (Olympus).

**RESULTS**

**VLP entry**

The schematic organization of the modified SFV replicon and the protocol used for the VLP production are summarized in Fig. 1(a) and (b), and were derived from Dorange et al. (2004). In this study, we sought to evaluate the cellular pathway leading to transgene expression after a single round of VLP transduction. VLP infectivity was measured using BHK-21 as target cells and by monitoring EGFP expression.

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**Fig. 1.** Constructions and sequential transfection. (a) Schematic structure of the modified SFV vector, for details see Dorange et al. (2004). CMV, Cytomegalovirus immediate-early promoter; RS, retroviral sequence; IRES, internal ribosomal entry site from Encephalomyocarditis virus; EGFP, enhanced green fluorescent protein. (b) On day 1 (D1) pMDG was transfected. On day 2 (D2), the modified SFV vector, expressing EGFP was transfected. Twenty-four or 36 h after the second transfection, the supernatant was harvested and filtered, and BHK-21 cells were transduced. (c) Un-palmitoylated mutants. (i) A single mutation (T2808G) was introduced in pMDG, which swaps a cysteine for a glycine (C489G). (ii) CMV-IE promoter-driven nsP1 expression vector. (iii) From this vector, we generated a palmitoylation-deficient mutant nsP1 form by removing three cysteines (AC418–C420 and Δnt1251–1260). (iv) To test the impact of palmitoylation ablation on VLP production, the mutated nsP1 sequence was introduced into an SP6 26Sm2 SFV vector.

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First, we confirmed the use of a VSV-G-mediated pathway by demonstrating complete inhibition of transgene expression when transduction was carried out in the presence of anti-VSV-G neutralizing antibodies (Fig. 2).

SFV VLPs have a composition that differs slightly from a classical virus, with no capsid protein and a high density of envelope protein at their surface (Dorange et al., 2004). We therefore sought to confirm that the entry pathway used by VSV-G was also used by the VLPs. To test whether the VLP infected cells via endosomes, we incubated the target cells with VLP in the presence of various concentrations of the acidification inhibitors bafilomycin A1 or chloroquine (Fig. 2) (Fischer et al., 2004). With these two compounds we observed a dose-dependent effect, with an almost complete block of entry at the higher concentrations of inhibitor. These experiments confirmed that the VLPs enter target cells via the endocytic pathway. As the two drugs exerted their effect on early endosomes, we next tested the effect of brefeldin A, a compound blocking maturation of the late endosome compartment (Fischer et al., 2004). Brefeldin A also reduced EGFP expression, although to a lesser extent, even at a high concentration (Fig. 2). These observations are in accordance with the known mechanism of VSV-G promoted membrane fusion that mainly relies on pH decrease, while brefeldin A affects endocytosis by disturbing the intracellular trafficking of endosomes (Yao et al., 2003).

**Transduction of HeLa cells**

Among the cell lines tested to evaluate the tropism of the VLPs, we included the well-characterized human epithelial cell line HeLa. Unexpectedly, these cells do not express the replicon when it is mobilized using VLPs. This observation might not reflect an envelope-specific entry problem as similar results were obtained when a modified SFV replicon was mobilized via retroviral particles bearing an amphotropic envelope (E. Piver & J.-C. Pages, unpublished data). Furthermore, HeLa cells express a functional VSV-G receptor, as confirmed by their susceptibility to transduction by VSV-G pseudotyped retroviruses (Fig. 2). Transfection of HeLa cells with the plasmid 26Sm2, encoding the SFV replicon under a CMV-IE promoter, did not give rise to EGFP expression (Fig. 3a). In addition, electroporation of HeLa cells using RNAs from an SFV

![Fig. 2. VLP entry. Effect of VSV-G antibodies: chloroquine, brefeldin A and bafilomycin A1 were tested at various concentrations in relation to infectivity of VLPs. BHK-21 cells were pre-incubated with the specific drug 1 h prior to transduction. The transduced cells were incubated for 24 h with the various reagents. VSV-G antibodies were added only during infection. The ratio of infection for each condition was obtained as follows: the titres calculated for each condition were divided by the titre in the absence of any compound multiplied by 100. To determine titres, we quantified the number of infected cells by flow cytometry. Data were obtained from three independent experiments in duplicate.](Image)

![Fig. 3. VLP expression in HeLa cells. (a) HeLa cells were transduced with an EGFP retroviral vector pseudotyped with the VSV-G (pBullet-pMN gag-pol-pMDG) or VLPs (26Sm2 SFV-pMDG). For comparison, BHK-21 cells were transduced with the same two supernatants. (b) Expression of EGFP in HeLa cells using plasmids where translation is IRES-dependent (pIRES2-EGFP) or IRES independent (pEGFP-C1). The 26Sm2 SFV vector is transfected for comparison. Cells were analysed using a flow cytometer (Becton Dickinson); fluorescence intensity is the mean fluorescence intensity for the cells above threshold.](Image)
replicon-expressing β-galactosidase, pSFV-3, leads to efficient transgene detection (data not shown). Other authors also efficiently expressed an SFV replicon within HeLa cells (Ahola et al., 2000; Salonen et al., 2003). An important difference between the latter constructs and our vector is the presence of an IRES to drive transgene expression in our vector. The presence of this sequence is required for transgene expression as the internal p26S promoter has been neutralized in the 26Sm2 construct. Transgene expression through internal translation initiation might be expected to be less efficient, as compared with the use of the 26S promoter, which drives substantial transcription when functional. This could explain why, even in permissive cells such as BHK-21, we observed only moderate levels of EGFP expression using the 26Sm2 construct. We therefore sought to evaluate the efficiency of the IRES in HeLa cells. This was tested using two commercially available EGFP expression vectors, one expressing EGFP from the CMV-IE promoter pEGFP-C1, the other expressing EGFP from the same promoter but with an IRES isolated from EMCV (pIRE2-EGFP). As shown in Fig. 3(b), there was a marked difference in the level of EGFP expression from the IRES, which reduced EGFP expression by approximately two orders of magnitude. As a control experiment, we tested the same two plasmids and also the 26Sm2 replicon in 293T cells (Fig. 3b). However, in these cells, the EGFP expression induced by 26Sm2 allowed clear detection of the transfected cells and therefore allowed us to follow the mobilized VLPs (Dorange et al., 2004).

Role of the palmitoylation in the subcellular localization of VLPs

To study the localization of the two main components of the VLPs, the SFV replication complex and the VSV-G envelope protein, we designed a series of constructs (Fig. 1c). The VSV-G contains an intracytoplasmic cysteine (C489), which is post-translationally acylated (Whitt & Rose, 1991). We introduced a mutation leading to the substitution of this cysteine with glycine (C489G) (nt T2808G), in order to prevent VSV-G palmitoylation [Fig. 1c(i)]. The phenotype resulting from this mutation was evaluated by confocal microscopy. As expected, the mutated VSV-G was still expressed at the cell surface (Fig. 4a and c). However, close examination revealed a wider distribution of the mutant protein as compared with a semi-clustered pattern for the wild-type. The more characteristic modification induced by the mutation was the marked reduction in the number and size of filopodia observed at the cell surface of expressing cells (Fig. 4a and c), bearing in mind that

![Fig. 4.](http://vir.sgmjournals.org) Confocal microscopy. Pictures represent superposition of all different sections. (a) Cells transfected with pMDG harbour numerous filopodia. The VSV-G protein, Alexia 488 staining, decorates the filopodia. (b) Expression of wild-type nsP1, Alexia 594, also induces filopodia formation at the cell surface. (c) Palmitoylation-deficient VSV-G, Alexia 488, is less potent for filopodia induction. (d) The palmitoylation-deficient nsP1, Alexia 594, is redistributed throughout the cytoplasm. No filopodia were observed. Co-transfection of individual vectors. Pictures are made from single sections of doubly transfected cells. (e) Co-transfection of the two wild-type expression vectors resulted in labelled filopodia at the membrane. In the overlay, the presence of yellow filopodia indicates a co-localization of VSV-G and nsP1. (f) For co-transfection of the mutated nsP1 and wild-type pMDG, we observed an nsP1 redistribution with VSV-G alone filopodia in green. (g) Expression of the palmitoylation-deficient VSV-G with the wild-type nsP1 showed filopodia and co-localization of the two proteins. (h) For palmitoylation-deficient VSV-G and nsP1 proteins the overlay picture suggests their co-localization.
293T cells are spontaneously prone to the formation of small filopodia.

It has recently been shown that the nsP1, together with nsP3 in a later phase, determine the localization of the SFV replication complex (Salonen et al., 2003). To investigate the co-localization of the SFV replication complex with VSV-G, we constructed an nsP1-expressing vector with three cysteines deleted (∆C418–C420, Δnt 1251–1260), which support nsP1 palmitoylation. After transfection, the wild-type and mutant proteins showed a completely different subcellular localization. Wild-type nsP1 was mainly found at the cell surface, while the mutated nsP1 was largely found in the cytoplasm (Fig. 4b and d). As previously found for the wild-type nsP1 by Ahola et al. (2000), we also observed that only cells transfected with the wild-type nsP1 plasmid generated a high number of filopodia. However, almost no secondary structures were observed with the mutated nsP1 (Fig. 4b and d) (Ahola et al., 2000).

We then assessed the co-localization of the wild-type and mutated proteins. The wild-type VSV-G co-localized with the wild-type nsP1 at the cell surface and, importantly, within filopodia (Fig. 4e, overlay). In contrast, the mutant nsP1 had almost no co-localization with the wild-type VSV-G within filopodia, and retained a cytoplasmic distribution (Fig. 4f). Although the mutant VSV-G C489G was widely distributed at the cell surface, it still gave rise to some co-localization with the wild-type nsP1, as discussed below (Fig. 4g). This had some functional implications (Table 1). Lastly, co-expression of the two mutated proteins led to some overlap in their distribution (Fig. 4h, overlay). The co-localization areas certainly resulted from the large cell size of the transfected cells. This observation indicates that the nsP1 palmitoylation is mandatory for SFV expression.

We then tested the mutated proteins for their ability to support VLP production. We first confirmed that the mutated VSV-G was still functional, using a retroviral pseudotyping assay (Table 1) (Whitt & Rose, 1991). Next the palmitoylation-deficient VSV-G was co-transfected with the 26Sm2 plasmid into 293T cells. Quantification of EGFP expression in target cells indicated that the SFV VLPs are produced at a titre almost identical to that obtained using wild-type VSV-G (Table 1). These results suggested that either the VSV-G is not the limiting factor in VLP production or that the amount of VSV-G present at the budding site was not dramatically altered by preventing VSV-G palmitoylation.

To address the role of the nsP1 in VLP formation, we cloned the mutated nsP1 into an SP6-dependent derivative of the 26Sm2 vector. After transfection we were able to detect neither EGFP expression nor particle formation as tested by the transduction of fresh cells with supernatant from transfected cells. This observation indicates that the nsP1 palmitoylation is mandatory for SFV expression.

**DISCUSSION**

Following the development of an efficient and safe method to mobilize SFV vectors using VLPs, we aimed to understand the cellular events involved in particle behaviour (Dorangé et al., 2004). First we looked at the pathways through which VLP enter target cells. Anti-VSV-G antibodies induced a complete block of transduction and inhibitors of endosome acidification reduced transduction by 90 %. Furthermore, brefeldin A, which alters cellular vesicle trafficking, was the least efficient of the compounds tested (Fig. 2), suggesting a rapid post-entry escape of VLPs from the endosomes. We can conclude that VSV-G, even when integrated into VLPs, maintains its natural fusogenic properties, regardless of the absence of an associated capsid. This is in agreement with results obtained using VSV-G coated liposomes (Abe et al., 1998).

Despite the wide tropism of the SFV VLPs, we noticed that HeLa cells were very poorly transduced. As it has been demonstrated that these cells express the VSV-G surface receptor, and that the SFV replicon was functional within these cells, we sought an alternative explanation for this observation. We studied whether the presence of an EMCV IRES influenced the expression of the transgene mobilized through the modified SFV vectors in HeLa cells. A similar observation has been reported for various cell lines by Mizuguchi et al. (2000). We addressed this question by using plasmids with or without IRES sequences between the promoter and the transgene (Fig. 3). Our data confirmed the inefficiency of the IRES in HeLa cells (Mizuguchi et al., 2000). In future experiments, and to circumvent this difficulty, it might be worthwhile measuring the availability of the cellular factors required by the IRES, although such

**Effect of palmitoylated domain mutations on particle infectivity**

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**Table 1.** Titre of VLPs produced using palmitoylation-deficient mutants

<table>
<thead>
<tr>
<th>Transfected sequence</th>
<th>Titre (transducing unit ml⁻¹)</th>
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<tbody>
<tr>
<td>pBullet-pMNag-pol-pMDG</td>
<td>1.22 × 10⁶</td>
</tr>
<tr>
<td>pBullet-pMNag-pol-pMDG-palm(−)</td>
<td>1.3 × 10⁶</td>
</tr>
<tr>
<td>26Sm2 SFV-pMDG</td>
<td>2.45 × 10⁵</td>
</tr>
<tr>
<td>26Sm2 SFV-pMDG-palm(−)</td>
<td>1.20 × 10⁵</td>
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factors have been described to be active in HeLa cells (Choi et al., 2004). Here, SFV-driven transcription shut-off might induce a rapid decay of these proteins, thus prohibiting IRES-dependent expression. The use of non-cytotoxic SFV vectors should prevent this side effect (Lundstrom et al., 2000; Perri et al., 2000). Alternatively, IRES activity is likely to be susceptible to structural problems in the context of the replicon.

A series of experiments was conducted in order to probe the mechanisms underlying VLP formation. Observations that it is not only VSV-G that is able to coat VLPs suggested passive recruitment of proteins exposed at the particle surface (Lebedeva et al., 1997; Rolls et al., 1994). However, in the absence of envelope, spontaneous production of SFV replicon-containing particles has not been described. Furthermore, our attempts to generate VLPs using the murine amphotropic or the foamy virus envelopes have been unsuccessful (data not shown). An explanation for these observations might come from the expression system that we employed, which was weaker than the SFV vector expressing the ecotropic envelope described by Lebedeva and collaborators (Lebedeva et al., 1997; Rolls et al., 1994). On the other hand, the SFV-G-expressing system that we used is not based on an SFV replicon (Dorange et al., 2004).

Moreover, we observed the formation of filopodia in cells expressing VSV-G, and some authors have described spontaneous release of vesicles from VSV-G-expressing cells (Fig. 4a and c) (Abe et al., 1998). Importantly, we and others have shown that a formation of a high number of filopodia is also induced by nsP1 (Ahola et al., 2000; Laakkonen et al., 1996). Significantly, VSV-G and nsP1 co-localized within these structures (Fig. 4c). Therefore, we hypothesize that the formation of the VLPs might result from a synergistic effect of the two proteins. To verify this hypothesis we tried to modify the VSV-G content of the filopodia by changing its surface distribution. For this we mutated a palmitoylated cysteine in the VSV-G intracytoplasmic domain [Fig. 1c (iii)]. Despite the redistribution of the VSV-G at the cell surface we noticed almost no reduction of release of VLPs (Fig. 4b and Table 1). We next attempted to study a palmitoylation-deficient SFV vector. However, mutation of nsP1 in this way destroyed the ability of the replicon to replicate. This was surprising since nsP1 contains, together with the palmitoylation site, another membrane association domain (Kaariainen & Ahola, 2002). Our data suggest that these two domains are not exchangeable. As nsP1 cysteine deletion within the replicon completely blocked its replication, it might be worthwhile to test cysteine substitutions, which have a milder effect on replication (A. Mertis, personal communication). Nonetheless, the cell surface is not the only site for VLP formation. Rolls et al. (1994) have shown, using an SFV replicon expressing the VSV-G under the transcriptional control of the 26S promoter, that the subgenomic RNA was efficiently packaged. This could result from VLP formation taking place in an intracellular compartment such as the cytoplasmic vesicles described by Ahola and co-workers (Ahola et al., 2000; Kujala et al., 2001). Apart from the site of VLP formation, another aspect of the process remains unclear. Which cellular molecules are involved in the release of VLPs? A computer based sequence analysis of the VSV-G and the four SFV nsPs did not reveal the presence of domains allowing the recruitment of members of the ubiquitin-like family of proteins recently described to trigger particle budding for several viruses (Morita & Sundquist, 2004). Therefore, the host factors mediating scission responsible for VLP release await identification.

In summary, the present report provides interesting data relevant to the design of SFV vectors that can be mobilized through VLPs. For future constructs, not only the transgene but also the sequence, i.e. the IRES, that drives transgene expression should be considered.

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