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Sendai virus budding in the course of an infection does not require Alix and VPS4A host factors

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Abstract

Closing the Sendai virus C protein open reading frames (rSeV-ΔC virus) results in the production of virus particles with highly reduced infectivity. Besides, the Sendai virus C proteins interact with Alix/AIP1 and Alix suppression negatively affects Sendai virus like particle (VLP) budding. Similarly, the Sendai virus M protein has been shown to interact with Alix. On this basis, it has been suggested that Sendai virus budding involves recruitment of the multivesicular body formation machinery. We follow, here, the production of SeV particles upon regular virus infection. We find that neither Alix suppression nor dominant negative-VPS4A expression, applied separately or in combination, affects physical or infectious virion production. This contrasts with the observed decrease of SV5 virion production upon dominant negative-VPS4A expression. Finally, we show that suppression of more than 70% of a GFP/C protein in the background of a rSeV-ΔC virus infection has no effect either on SeV particle production or on virus particle infectivity. Our results contrast with what has been published before. Possible explanations for this discrepancy are discussed.

Keywords: Paramyxovirus; Sendai virus; Virus particle; Budding; Multivesicular bodies; Alix; VPS4A

Introduction

Enveloped viruses acquire their lipid by-layer by budding from cellular membranes. The virus particle components first assemble at these membranes in an orderly way before the modified portion of the membrane forms a bud that eventually pinches off. This process has been recognized similar to that of cellular vesicles budding into or out of multivesicular bodies (MVBs) owing to the fact that cellular partners involved in this vesiculation are recruited by proteins of several enveloped viruses. Retroviral Gag proteins and the matrix proteins of some negative strand RNA viruses have been shown to interact with these cellular partners via domains called late domains (Freed, 2002; Morita and Sundquist, 2004).

Different types of late domains have been characterized. P(T/S)AP, PPxY and YP(x)$_n$L late domains have been identified in the p6, the p2b and the p9 regions of respectively the human immunodeficiency virus type 1 (HIV-1), the Rous sarcoma virus (RSV) and the equine infectious anemia virus (EIAV) Gag proteins (Gottlinger et al., 1991; Huang et al., 1995; Wills et al., 1994; Xiang et al., 1996; Puffer et al., 1997). As for the negative-strand RNA viruses, the same P(T/S)AP and PPxY late domain sequences have been identified in the matrix M proteins of, respectively, the vesicular stomatitis virus (VSV), a *Rhabdovirus*, and the Ebola virus, a *Filovirus*, and have been shown to be relevant for their respective budding (Craven et al., 1999; Harty et al., 1999, 2000; Jayakar et al., 2000; Licata et al., 2003; Martin-Serrano et al., 2001, 2004). For the *Paramyxovirus* SV5, the matrix protein lacks these previously defined late domains, but rather contains a new motif (FPIV), of which the proline was found critical for its function (Schmitt et al., 2005). Interestingly, when this proline was mutated, leading to a

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recombinant SV5 virus affected in its multiplication, a compensatory mutation occurred rapidly, which restored budding. This mutation consisted in a new proline residue elsewhere in the matrix protein, recreating the more general motif ϕ-P-x-V.

These late domain motifs are likely to function by recruiting the cellular machinery involved in MVB formation via interactions with cellular partners which are part of this machinery (Slagsvold et al., 2006). P(T/S)AP late domain mediates binding to TSG101 (Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001) and YP(x)nL appears to bind to AIP1/Alix (Martin-Serrano et al., 2003a,2003b; Strack et al., 2003; Vincent et al., 2003; von Schwedler et al., 2003). For PPxY, the cellular partners relevant to budding have yet to be unequivocally identified, although PPxY motif has been shown to interact with Nedd4-related E3 ubiquitin ligases (Harty et al., 1999; Kikonyogo et al., 2001; Timmins et al., 2003), an interaction that may, in turn, allow recruitment of proteins involved in MVB formation (Strack et al., 2003). The cellular partner of the SV5 ϕ-P-x-V motif has not been determined. Regardless of the nature of the cellular partner, the cellular machinery involved in MVB formation appears to require the activity of the VPS4A ATPase. VPS4A is required to recycle this machinery allowing multiple rounds of vesicle formation and its inactivation results in the blocking of the vesiculation (Babst et al., 1997, 1998; Bishop and Woodman, 2001; Katzmann et al., 2002). Dominant negative versions of VPS4A (DN-VPS4A) are available whose overexpression blocks budding of *Retroviruses*, *Filoviruses* and *Paramyxoviruses* (Garrus et al., 2001; Licata et al., 2003; Martin-Serrano et al., 2003a,2003b; Shehu-Xhilaga et al., 2004; von Schwedler et al., 2003; Schmitt et al., 2005), with the exception of VSV. For this latter, despite the clear demonstration of the presence of a functional late domain PPPY in the matrix protein (Irie et al., 2004a; Craven et al., 1999), despite the fact that this domain could be exchanged with that of the Ebola VP40 protein (Irie et al., 2005), DN-VPS4A overexpression had no deleterious effect on its budding (Irie et al., 2004b).

For Sendai virus (SeV), another member of the *Paramyxovirus* family, the viral protein claimed to recruit the MVB sorting machinery has first been an accessory protein named C. The C protein is in fact part of an array of four C polypeptides encoded by the viral P mRNA in the +1 open reading frame (Curran and Kolakofsky, 1990). The C proteins have been shown to regulate the activity of the viral RNA polymerase (Cadd et al., 1996; Latorre et al., 1998; Tapparel et al., 1997). Moreover, they have been found to counteract the interferon-mediated antiviral innate response (Takeuchi et al., 2001; Gotoh et al., 1999; Garcin et al., 1999, 2000, 2001, 2002, 2004). C protein knock out viruses (rSeV-ΔC) were found highly attenuated in mice and produced virus particles with highly decreased infectivity to particle ratios (Latorre et al., 1998; Kurotani et al., 1998). Moreover, C expression was shown to moderately facilitate (2.5-folds) SeV M shedding, when M was expressed along with the other viral proteins N, F and HN. Under these conditions, however, C expression had no noticeable effect on the production of the three other proteins (Sugahara et al., 2004). The C protein was further shown to interact with AIP1/Alix (Sakaguchi et al., 2005; Irie et al., 2006), and Alix overexpression resulted in a 4-fold increase of M in virus particles and its suppression lowered this M amount by less than 3-folds (Sakaguchi et al., 2005). This contrasted with the absence of M budding enhancement in virus like particles (VLP) after Alix overexpression (Irie et al., 2007). Overexpression of a DN-VPS4A was shown to lower the SeV infectivity produced in the cell medium by about 4-folds when compared to the condition of wt-VPS4A expression (Sakaguchi et al., 2005). Curiously, when the same authors performed SeV virus like particle (VLP) budding assays by expressing M, N, F, HN and C, they found that overexpressing VPS4A-wt, this time, caused a strong suppression of VLP release, a release that was rather restored by DN-VPS4A expression. These various observations, despite some inconsistency, led to propose that the C proteins participate in virus particle production by recruiting the MVB formation machinery.

On the other hand, SeV M protein has clearly been identified as central for virus particle production without which no particles are formed (Sakaguchi et al., 1999; Takimoto et al., 2001; Sugahara et al., 2004; Mottet-Osman et al., 2007 and unpublished results). Very recently, a YLDL domain was identified which is claimed to promote a specific M–Alix interaction, and suppression of Alix was shown to result in a 10-fold M shedding decrease (Irie et al., 2007). YLDL, however, could not be replaced by other late domains and, when M was co-expressed with N, HN and F, the 2-fold increase in M shedding, promoted by the C expression, was not observed for the other three proteins.

In the end, the observations concerning SeV budding in relation with the MVB machinery are overall difficult to interpret. In this study, we have followed the effects that suppression of AIP1/Alix and DN-VPS4A overexpression had on virus particle budding during the course of a regular SeV infection. Unexpectedly, we were not able to demonstrate a significant inhibitory effect on physical or infectious particle production under either condition. Even a coupling of the two conditions did not lead to a more relevant effect. We were equally not successful in demonstrating the effect of Alix suppression and DN-VPS4A expression on M shedding when the protein was expressed by itself in transfected cells. In the end, we measured virus particle production after suppression of a C protein expressed from an otherwise delta C protein recombinant SeV (rSeV-ΔC-GFP/C). Here again, suppression of C was not followed by a decrease in virus particle production. The involvement of the MVB machinery in SeV particle budding is discussed in view of these data.

Results

Suppression of AIP1/Alix during the course of a viral infection

In a first series of experiments, suppression of Alix was achieved by transfection of cells with siRNAs. Different cell types were used (HeLa, 293T) and were transfected with the appropriate siRNAs 48 h prior to SeV infection. Virus

particles were monitored at different times after infection by measures of the physical particles produced or by titration of the viral infectivity in the supernatant. Although Alix suppression, monitored by Western blotting, was effective, under no conditions its suppression had an effect on virus particle production estimated by the measure of the N protein production (see Fig. 1, for an example).

As these experiments are dependent on fluctuating transfection efficiencies (varying between 30–70%), the effect of suppression of Alix on virus particle budding could have been masked by the viral production of the non-transfected but infected cells. In order to make up for this problem, cell lines were produced that constitutively expressed α-Alix-siRNAs in all the cells (>95%). These were generated by transduction of the cells with a lentivirus vector constitutively expressing the α-Alix-siRNAs along with the GFP protein. This latter marker served the purpose of selecting the transduced cells, so to achieve a culture in which all the cells were actually expressing the siRNAs. Two cell lines, FTGH and 293T, were prepared in this way with their matched control, i.e. cell lines transduced with a vector expressing GFP only.

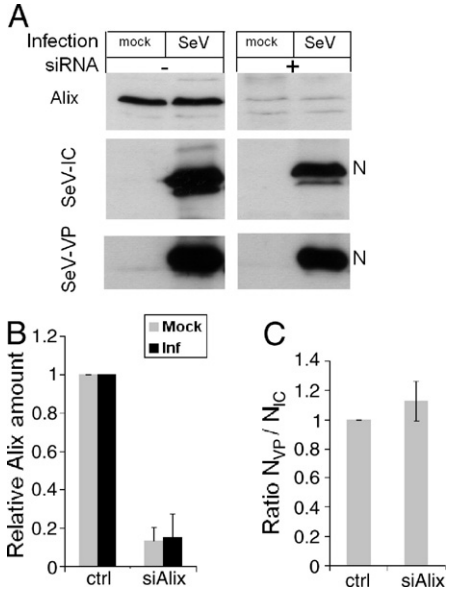

Fig. 1. Cellular Alix suppression by siRNA transfection and SeV particle production. HeLa cells in 35 mm-diameter dishes were transfected with 20 μM of anti-Alix siRNA, and 48 h later, the cells were infected with SeV (m.o.i. of 2). Forty hours later, culture medium and cells were harvested. Cell extracts were prepared and the virus particles present in the supernatants were isolated as described in Materials and methods. (A) Forty micrograms of total proteins (measured by Bradford assay) of the cell extracts or about 1/6th (adjusted to the total amount of proteins in cell extracts) of the virus particles (VP) were analyzed by Western blotting using α-Alix, α-SeV-N antibodies. (B) The bands of Alix in cell extracts were quantified and expressed relative to the values obtained in control cells (minus siRNA) normalized to 1. (C) The bands of N proteins in cell extracts and virus particles were quantified and expressed relative to the values obtained in control cells normalized to 1. Budding efficiency was estimated by following the N protein fate (ratio N_{VP}/N_{IC}) and was calculated by dividing the relative N signal obtained in VP by the relative signal obtained in cell extracts. For (B) and (C), bars indicate standard deviation from the mean (3 experiments).

FTGH or 293T cells expressing α-Alix-siRNAs were then infected or mock infected with SeV and the cells and the supernatants were collected. The amounts of Alix, of actin and of the viral proteins HN, N and M present in the cell extracts were monitored by Western blots. The virus particles present in the cell supernatants were collected and similarly analyzed for their content in the same viral proteins. The results, presented in Fig. 2, show that the suppression of the Alix protein was efficient in each sample derived from cells expressing the α-Alix-siRNAs (+) (Fig. 2E). Conditions of Alix suppression had no effect on the amounts of viral proteins present in the cell extracts, indicating that the extent of the infection was similar, although a general increase was observed between 16 and 24 h pi (for the FTGH-cells). As for the virus particle production (Fig. 2B, SeV-VP), here again, no difference could be observed between the ±siRNA conditions (Figs. 2C and D, for quantification).

A fraction of the cell supernatants was used to measure the infectious titers of the produced particles (Table 1). The titers obtained varied somehow from experiment to experiment, as did the comparison between the ±Alix conditions. A 2-fold decrease could be observed in some experiments (Exp 1, Exp 5, 48 h pi), but this was not consistent. For instance, no difference could be noted in Exp 2 and 4 (16 h and 48 h pi) and cases of increase virus production were also observed (Exp 2 and 3, 24 h pi). Matching these results with those obtained by Western blots indicates that suppression of Alix in both cell types had no significant effect on virus particle production.

Expression of VPS4A dominant negative proteins and SeV particle production

Next, the effect of neutralizing the action of VPS4A by overexpression of two dominant negative (DN) versions (E228Q and K173Q) was analyzed. 293T cells were transfected with plasmid expressing the wt or the mutant versions of the protein prior to infection with SeV. Forty hours following infection, the cells and cell supernatants were collected and analyzed as above. In this experiment, the VPS4A proteins are fused with the GFP protein so that the efficiency of transfection was readily estimated by the amount of fluorescent cells. In the experiment presented in Fig. 3 (and others, not shown), the transfection efficiency was close to 65%. Western blots show the expression of the VPS4A proteins (Fig. 3A, VPS4A). Their expression did not alter the amounts of viral proteins monitored in the cells extracts (Fig. 3A, SeV-IC). In Fig. 3B, the amounts of proteins in the virus particles produced are presented. Neither overexpression of VPS4A (wt lane), nor expression of the DN-VPS4A had a marked effect on virus production (Fig. 3C for quantification). This lack of effect was corroborated by the titrations of the virus particles presented in Table 2. In this case the results were very steady. If anything, a slight increase in infectious virus was observed when the DN mutant K173Q was present. This variation, however, remained nonsignificant.

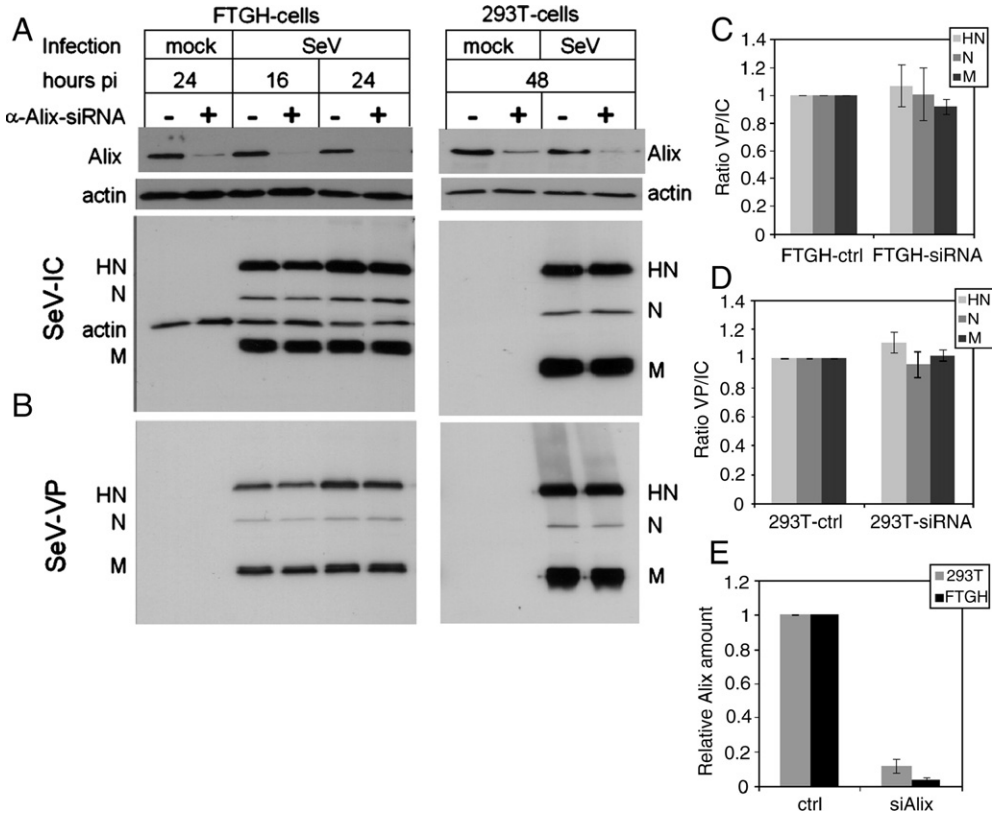

Fig. 2. Cellular Alix protein suppression by siRNA expression and SeV particle production. FTGH-ctrl, FTGH-α-Alix-siRNA, 293T-ctrl or 293T-α-Alix-siRNA cell lines in 35 mm-diameter dishes were infected with SeV (m.o.i. of 2). Culture medium and cells were harvested at the indicated time post infection (hours pi). Cell extracts were prepared and the virus particles present in the supernatants were isolated as described in Materials and methods. (A) Forty micrograms of total proteins (measured by Bradford assay) of the cell extracts or (B) about 1/6th (adjusted to the total amount of proteins in cell extracts) of the virus particles (VP) were analyzed by Western blotting using α-Alix, α-actin, α-SeV-HN, α-SeV-N and α-SeV-M antibodies. (C) The bands of SeV-HN,-N and-M proteins in FTGH cell extracts and virus particles were quantified and expressed relative to the values obtained in control cells normalized to 1. Budding efficiency (ratio VP/IC) as in Fig. 1C. Standard deviations from the mean are indicated (3 experiments). (D) As in C but with 293T cells (3 experiments). (E) The bands of Alix in FTGH or 293T cell extracts were quantified and expressed relative to the values obtained in control cells normalized to 1 (6 experiments). Standard deviations, as in (C) and (D).

Combining Alix suppression and DN-VPS4A expression

Assuming that Alix suppression or DN-VPS4A expression effects could be masked because a too high fraction of cells would not be affected by one or the other condition, we reasoned that the combination of the two conditions could complement for each other possible defection. The 293T cells expressing the α-Alix-siRNAs and their matched control were therefore transfected with the plasmids expressing the VPS4A protein and its DN versions in a protocol similar to that described in Fig. 3. The results shown in Fig. 4 are very clear in that they do not show any sign of additive effects of the two combined conditions, this in situations where Alix and the VPS4A proteins were, respectively, effectively suppressed and efficiently expressed. Once more, titration of the infectious particles in the supernatants came to corroborate this conclusion (Table 3).

Table 1
Cellular Alix protein suppression and SeV infectious particles production

	Cells	α-Alix-siRNA expression	pfu at 16 hours pi	pfu at 24 hours pi	pfu at 48 hours pi
Exp1	FTGH	–	3.9×10^5	nd	nd
		+	1.4×10^5	nd	nd
Exp2		–	4.5×10^5	2.5×10^6	nd
		+	4.5×10^5	3.4×10^6	nd
Exp3		–	nd	1.2×10^6	nd
		+	nd	2.1×10^6	nd
Exp4	293T	–	nd	nd	2.4×10^6
		+	nd	nd	2.1×10^6
Exp5		–	nd	5.7×10^5	9.8×10^6
		+	nd	3.5×10^5	4.1×10^6

FTGH-ctrl, FTGH-α-AIP1-siRNA, 293T-ctrl or 293T-α-Alix-siRNA cell lines were infected with SeV (m.o.i. of 2). Culture mediums were harvested at the indicated time post infection and viral titers were determined as described in Materials and methods.

Budding of SeV M in relation to Alix and VPS4A functions

The main purpose of this study was to follow the production of the SeV virus particle in the context of an infection. We felt, however, disturbing that differences in SeV-virion production could not be observed under conditions previously reported to negatively affect SeV-virus like particle (VLP) production. Following the recent report that the SeV-M budding was affected by Alix suppression (Irie et al., 2007), we decided to drift from our original goal. We performed the analysis of M

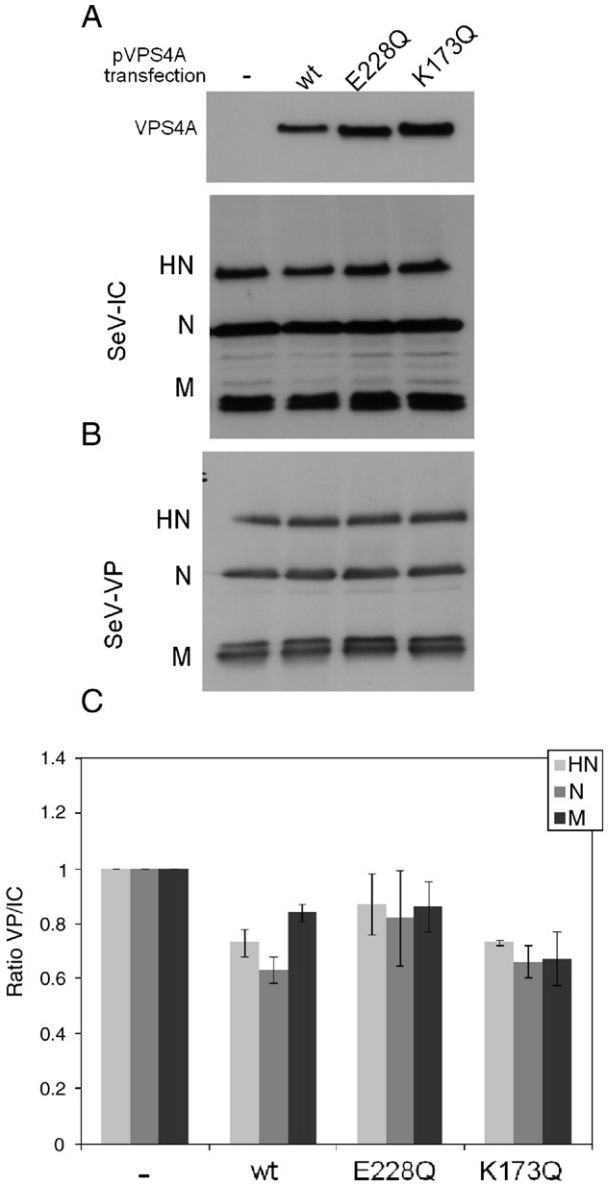

Fig. 3. Cellular VPS4A protein expression and SeV particle budding. 293T cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q using the FuGENE 6 transfection reagent (Roche). Six hours after transfection, cells were infected with SeV (m.o.i. of 2). Culture medium and cells were harvested at 40 h pi. Cell extracts were prepared and the virus particles present in the supernatants were isolated as described in Materials and methods. (A) Forty micrograms of total proteins (measured by Bradford assay) of cell extracts or (B) about 1/6th (adjusted to the total amount of proteins in cell extracts) of the virus particles (VP) were analyzed by Western blotting using anti-GFP, anti-SeV HN, anti-SeV N and anti-SeV M antibodies. (C) The bands of SeV-HN, -N and -M proteins in cell extracts and virus particles were quantified and expressed relative to the values obtained in control conditions (absence of transfected VPS4A) normalized to 1. Budding efficiency (ratio VP/IC) as in Fig. 1C. Standard deviations from the mean are indicated (4 experiments).

budding when M was expressed alone in the context of the DN-VPS4A, as well as in conditions of Alix suppression. As shown in Fig. 5, neither condition could exert any significant influence on the amount of M shed into the medium in the form that sediments through a 25% glycerol cushion as virus particles do. In part A (VPS4A) and in part B (Alix suppression), the ratios of intracellular to extracellular M (HA-M_{IC}/HA-M_{OUT}) remain unchanged. It is noteworthy that the M protein tagged at the N-terminus with an HA epitope was used rather than the wild type M. However, this is unlikely to explain the lack of effect of these conditions, since the HA-M was previously shown to behave exactly like the wild type protein in the course of an infection (Mottet et al., 1996). In the end, the study of SeV budding, reduced to that of its main effector, did not lead more than that of its complete set of structural proteins to conclude in the involvement of MVB machinery partners.

Expression of VPS4A dominant negative proteins reduces SV5 particle production

At this point, it became important to check whether the conditions of our experiments were sensitive enough to demonstrate an effect of the applied conditions on virus particle budding. We therefore attempted to reproduce the data obtained in the study of the SV5 virus, a *Paramyxovirus*, genus *Rubulavirus*, for which it was reported that the budding of virions from infected cells was affected by the expression of the DN-VPS4A proteins (Schmitt et al., 2005). SV5 budding (Fig. 6A) was then followed in parallel with that of SeV (Fig. 6B). In Figs. 6A and B (VPS4A), the extent of VPS4A protein expression is shown. Below, the extent of the SV5 or SeV infections is estimated after immune-precipitation of two viral proteins, HN and N (cell extracts). The lower panel shows that the viral particles recovered in the supernatants. In the case of SV5, the amount of virus particles (VP) was clearly diminished in co-expression with the two DN-VPS4A. Interestingly, the decrease in virus particle production was accompanied, in the infected cells, by a surplus of HN, likely reflecting its accumulation due to restricted virus particle production. Also remarkable is the presence of VPS4A in SV5 virus particles (identified by Western blotting, not shown). None of these features is seen in the case of SeV (Fig. 6B). The amount of intracellular HN is steady (cell extracts), as are the amounts of SeV particle

Table 2
Cellular VPS4A proteins expression and SeV infectious particles production

SeV	VPS4A	pfu at 40 h pi
Exp1	+wt	1.0×10^7
	+E228Q	1.5×10^7
	+K173Q	1.8×10^7
Exp2	+wt	7.7×10^7
	+E228Q	7.7×10^7
	+K173Q	9.0×10^7
Exp3	+wt	1.2×10^7
	+E228Q	1.4×10^7
	+K173Q	1.5×10^7
Exp4	No	4.6×10^7
	+wt	2.2×10^7
	+E228Q	2.5×10^7
	+K173Q	1.8×10^7

293T cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q. Six hours after transfection, cells were infected with SeV (m.o.i. of 2). Viral titers, as in Table 1.

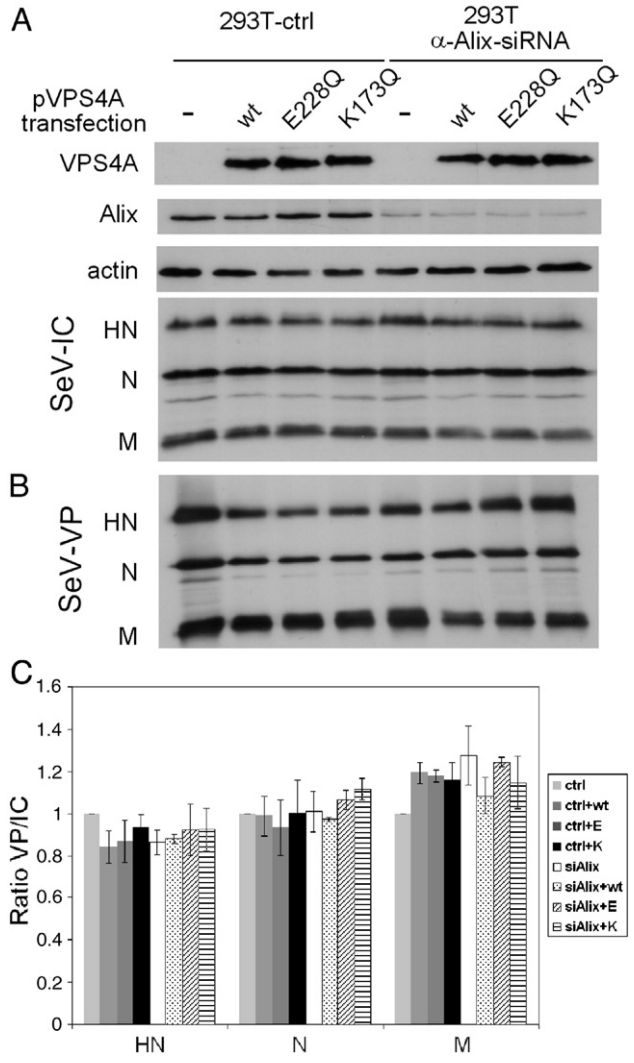

Fig. 4. Combined effect of Alix suppression and VPS4A proteins expression on SeV particle budding. 293T-ctrl and 293T-α-Alix-siRNA cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q. Six hours post transfection, cells were infected with SeV (m.o.i. of 2). Culture medium and cells were harvested at 40 h pi. Cell extracts were prepared and the virus particles present in the supernatants were isolated as described in Materials and methods. (A) Forty micrograms of total proteins in cell extracts or (B) about 1/6th (adjusted to the total amount of proteins in cell extracts) of the virus particles (VP) were analyzed by Western blotting using anti-GFP, anti-SeV HN, anti-SeV N and anti-SeV M antibodies. (C) The bands of SeV-HN, -N and -M proteins in cell extracts and virus particles were quantified and expressed relative to the values obtained in control conditions (293T-ctrl cell lines in absence of transfected VPS4A) normalized to 1. Budding efficiency (ratio VP/IC), as in Fig. 1C. Standard deviations from the mean are indicated (2 experiments).

recovered in the supernatants (VP). In this particular comparison, the proteins were radiolabeled so that the evaluation of their amounts in virus particles was direct. The same decrease in SV5 proteins in virus particle could be observed by Western blot analysis (not shown). It is also noteworthy that radio-isotope labeling conditions were also performed for the experiments presented in Figs. 2–4 and, as in the present case, these conditions never led to the observation of a SeV budding decrease (not shown). Production of SV5 infectious particles was also estimated by titration and a minimum of a 3-fold decrease in virus titer could be observed (Table 4). In the end, our ability to reproduce the results obtained for SV5 validates our observations concerning SeV.

SeV particle production in the context of the C protein suppression

The involvement of the cellular MVB machinery in SeV particle production has been called upon after the observation that (1) recombinant SeVs, in which the C protein ORFs had been closed (rSeV-ΔCs), were highly attenuated and produced virus particles with significant lower infectivity (Kurotani et al., 1998; Latorre et al., 1998), and (2) the SeV-C and M proteins were shown to interact with Alix (Sakaguchi et al., 2005; Irie et al., 2007). Recombinant SeV-ΔCs are debilitated viruses that were rescued after several infection cycles in embryonated eggs (Latorre et al., 1998; Kurotani et al., 1998). This procedure gave potential room for selection of compensatory mutations. In that respect, the expression of a C protein (fused to the green fluorescent protein; GFP/C) from a supplementary transcription unit located between the F and the M genes of SeV-ΔCs (creating rSeV-ΔCs-GFP/C), was able to fully complement the ablation of the C proteins (Machiko Nishio, Daniel Kolakofsky, unpublished, University of Geneva). We rationalized, then, that suppression of the GFP/C protein in rSeV-ΔCs-GFP/C infection represented a more direct way to realize and analyze the ΔC protein conditions. To suppress the GFP/C protein, we made use of an A549 cell line constitutively expressing an siRNA directed against the GFP mRNA sequence (α-GFP-siRNA). This cell line, A549-LV-siGFP, was found very efficient in suppressing the M protein when its messenger carried the GFP-siRNA target sequence in its 3′ untranslated region, a modification introduced in a SeV recombinant called rSeV-M-gfpt (Mottet-Osman et al., 2007).

A549-LV-siGFP and its matched control cell line were then infected with rSeV-ΔCs-GFP/C. Both cell lines were also infected with rSeV-M-gfpt as a control to evaluate the potency of the α-GFP-siRNAs. From 16 h pi, the cells were metabolically radiolabeled and at 40 h pi, the cells and the supernatants were collected. The intracellular P, GFP/C, M and C proteins were detected by Western blots (Fig. 7A). In parallel the ^{35}S-labeled P, HN, F0 and N proteins were recovered by immune-precipitation from the cell extracts (Fig. 7B) and the

Table 3
Combination of Alix suppression and VPS4A proteins expression on SeV infectious particles production

VPS4A	293T-ctrl	293T-α-Alix-siRNA
+wt	$5.20\pm0.53\times10^6$	$3.73\pm0.18\times10^6$
+E228Q	$5.25\pm0.25\times10^6$	$4.27\pm0.29\times10^6$
+K173Q	$5.40\pm0.40\times10^6$	$4.37\pm0.42\times10^6$

293T-ctrl and 293T-α-Alix-siRNA cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q. Six hours after transfection, cells were infected with SeV at an m.o.i. of 2. Viral titers, as in Table 1. The results are presented as the mean of 2 experiments with standard deviation from the mean.

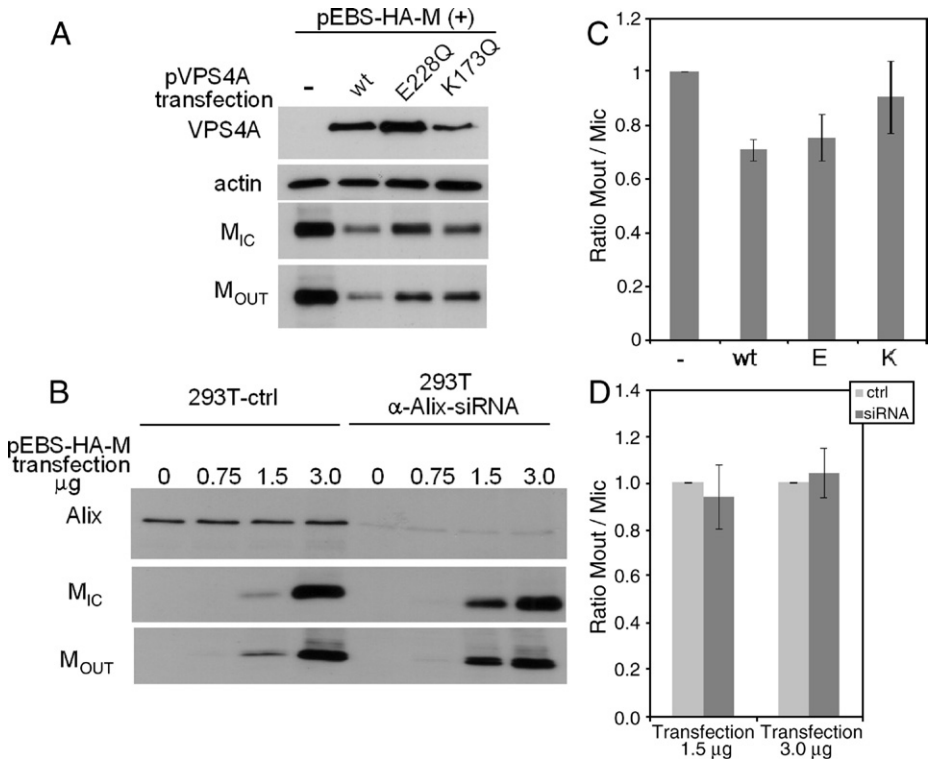

Fig. 5. Effects of VPS4A proteins expression and Alix suppression on SeV-M shedding. (A) 293T cells in 35 mm-diameter dishes were first transfected with 2 µg of pVPS4A-wt, pVPS4A-E228Q or pVS4A-K173Q, and 16 h later, transfected with 2 µg of pEBS-HA-M. Culture medium and cells were harvested at 40 hours post transfection. Cell extracts were prepared and the M present in the supernatants was isolated as described in Materials and methods. Forty micrograms of total proteins in cell extracts or about 1/6th of VP (adjusted to the total amount of proteins in cell extracts) were analyzed by Western blotting using anti-GFP and anti-HA antibodies. (B) 293T-ctrl and 293T-α-Alix-siRNA cells in 35 mm-diameter dishes were transfected with pEBS-HA-M (from 0 to 3 µg) as in (A). (C) The bands of SeV-HA-M in cell extracts and supernatants were quantified and expressed relative to the values obtained in control conditions (no VPS4A transfection) normalized to 1. Budding efficiency (ratio M_{OUT}/M_{IC}) was calculated by dividing the relative signal obtained in supernatants by the relative signal obtained in cell extracts. Standard deviations from the mean are indicated (2 experiments). (D) As in (C), except for the control conditions which correspond to 293T cells not expressing the siRNAs. M_{IC}: intracellular M; M_{OUT}: M in the culture medium.

radiolabeled viruses were collected from the supernatants and directly analyzed (Fig. 7C). Fig. 7A, rSeV-ΔCs-GFP/C and rSeV-M-gfpt lanes, shows the effect of α-GFP-siRNAs on, respectively, the GFP/C and the M levels (compare −/+ lanes). Note the absence of the C protein bands in the rSeV-ΔCs-GFP/C lanes, replaced by the GFP/Cs. Note as well the equivalent amounts of P detected which support the similar extent of infections of both viruses in both cell lines (compare rSeV-M-gfpt and rSeV-ΔCs-GFP/C, −/+ lanes). This point can be better made in Fig. 7B, in which the intracellular amounts of ^{35}S-labeled P, HN, F0 and N proteins are shown. Despite the overlap of N and F0 of the rSeV-ΔCs-GFP/C virus (the N protein is of different origin in the two viruses, Garcin et al., 1998), it is apparent that the extent of the infections in the ±α-GFP-siRNA expressing cell lines is equivalent (if anything the infection in the control cell line is a little higher). Panel C shows the virus particles produced. As expected from the M central role in virus particle formation, M suppression drastically decreased virus particle production (compare lanes 3 and 4, see also panel D, for quantification). On the other hand, GFP/C suppression led to no significant change in virus particle production. If anything, a slight increase can be observed which parallels the slight decrease of the intracellular virus proteins (compare lanes 5 and 6). Therefore a reduction in more than 70% the C protein did not affect SeV particle production (Fig. 7E, for quantification). The infectivity of the produced particles paralleled their physical production ($0.5\pm0.2\times10^6$ and $1.2\pm0.0\times10^6$, for respectively the control and the α-siGFP conditions). In conclusion, this experiment does not support an essential participation of C in SeV particle production, unless the remaining 30% of GFP/C is sufficient to provide a full C function.

Discussion

In this study we concentrated our efforts on examining the cellular parameters described before as participating in the formation and budding of SeV particles, i.e. the Alix and the VPS4A proteins, both partners of the MVB sorting machinery. Our experimental approach and tools have not been different from those used in the field with the exception of cell lines constitutively expressing si-RNAs to suppress the Alix and the GFP/C proteins. We have also been focusing almost exclusively on the virus particle production resulting from regular viral infections. The data we obtained could not confirm the conclusions reached before concerning the involvement of the MVB machinery.

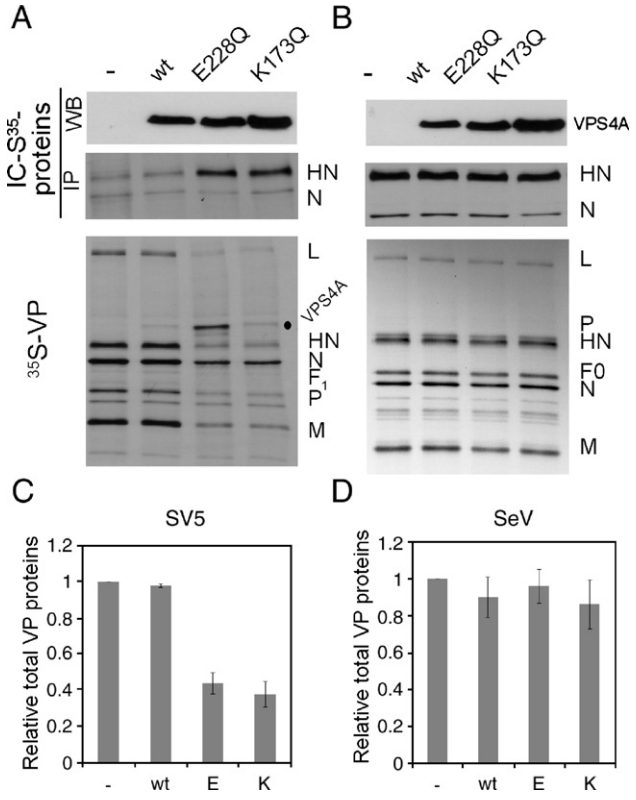

Fig. 6. Effect of VPS4A expression on SV5 and SeV budding. 293T cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q. Six hours after transfection, cells were infected with SV5 (A) or SeV (B) at an m.o.i. of 1 and radiolabeled from 16 h to 40 h pi. Culture medium and cells were harvested at 40 h pi. Cell extracts were prepared and the virus particles present in the supernatants were isolated as described in Materials and methods. Forty micrograms of cell extract proteins was used for detecting the VPS4A proteins by Western blot. The remaining fractions of the cell extracts were used to immune precipitate SV5 or SeV HN and NP and 1/10th of the total IP was analyzed by SDS-PAGE. About 1/6th (fraction adjusted to the total protein amount in cell extracts) of the virus particle samples (VP) were analyzed by SDS-PAGE. The radioactive gels were treated for autoradiography. (C and D) The bands of viral proteins in virus particles were quantified. Budding efficiency (relative total VP proteins) was calculated as the sum of the signals corresponding to the virus proteins and expressed relative to the control conditions (no VPS4A transfection) normalized to 1. Standard deviations from the mean are indicated [SV5 (C), SeV (D), 2 and 3 experiments, respectively].

We have also not been able to confirm the participation of the viral C protein in the process.

Alix suppression was performed using cell lines generated by a lentivirus vector expressing the α-Alix-siRNAs along with the GFP protein. GFP expression allowed selection and enrichment of the transduced cells. It also constituted a mean to evaluate the percentage of cells expressing the siRNAs at the time of the experiment. In our experiments, more than 95% of the cells were GFP positive, a feature that allowed us to get around the problem raised by a too low percentage of cells harboring suppression (as upon siRNA transfection) in front of 100% of infected cells. When we used the same approach to suppress the viral M protein, suppression was efficient enough to lower virus particle production by more than 50-folds (Gosselin-Grenet et al., 2006; Mottet-Osman et al., 2007). It is then under similar conditions of effective Alix suppression (as also attested by Western blot analysis, Figs. 2A and E) that we were not able to demonstrate a significant effect of this suppression on SeV particle production.

The effects of DN-VPS4A overexpression could not be evaluated under such optimal conditions. However, because the VPS4A proteins were fused to GFP, we could estimate the percentage of cells expressing these proteins by UV microscopy. We then only took into account the experiments in which more than 65% of the cells exhibited GFP fluorescence and therefore expressed the VPS4A proteins. Also, Western blots came to confirm the efficient expression of these proteins. Finally, we showed that interdicting with VPS4A function resulted in a decrease of SV5 particle production upon a regular virus infection, reproducing previously published data (Schmitt et al., 2005). This success came as a validation of our experiments since lack of demonstrated effects could not necessarily mean lack of effects. It is noteworthy, to mention at this point, that the VPS4A proteins were not incorporated in SeV particles as they were in SV5's, alluding to a dissimilar implication of these proteins between the two infections.

Interaction of C and Alix has been convincingly demonstrated by co-immune precipitation and by double hybrid interaction assays (Sakaguchi et al., 2005; Irie et al., 2007). This interaction has been shown to involve amino acids 212–357 of Alix (868 aa), and the last 10 amino acids of C. This interaction has received support in our experiments by the demonstrated ability of C to re-localize Alix at the plasma membrane (Dominique Garcin, not shown). The involvement of C in SeV budding, in relation with its association with Alix, however, remains a question of debate. As mentioned, the C proteins exert different actions during SeV infections, and this spectrum of effects complicates the interpretation of the C knocking out mutants. In our hands, suppression of more than 70% of C (GFP/C), failed to show a deficit in SeV particle production, and in virus particle infectivity, in contrast to what had been observed before (Kurotani et al., 1998; Latorre et al., 1998). Either, in our experiments, the remaining C can fully provide the C budding function, or the impairment of infectious SeV particle production observed before resulted from a deleterious effect of the IFN system, from a deregulation of viral RNA synthesis or from an unknown feature selected in the process of rescuing a poorly viable virus.

The recent observation that M interacts with Alix (Irie et al., 2007) introduces another level of complexity in understanding

Table 4
Effect of VPS4A proteins expression on SV5 infectious particle production

Virus	VPS4A	pfu at 40 h pi
SV5	+wt	4.5×10^5
	+E228Q	1.5×10^5
	+K173Q	1.4×10^5

293T cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q. Six hours after transfection, cells were infected with SV5 (m.o.i. of 1). Viral titers as described in Materials and methods.

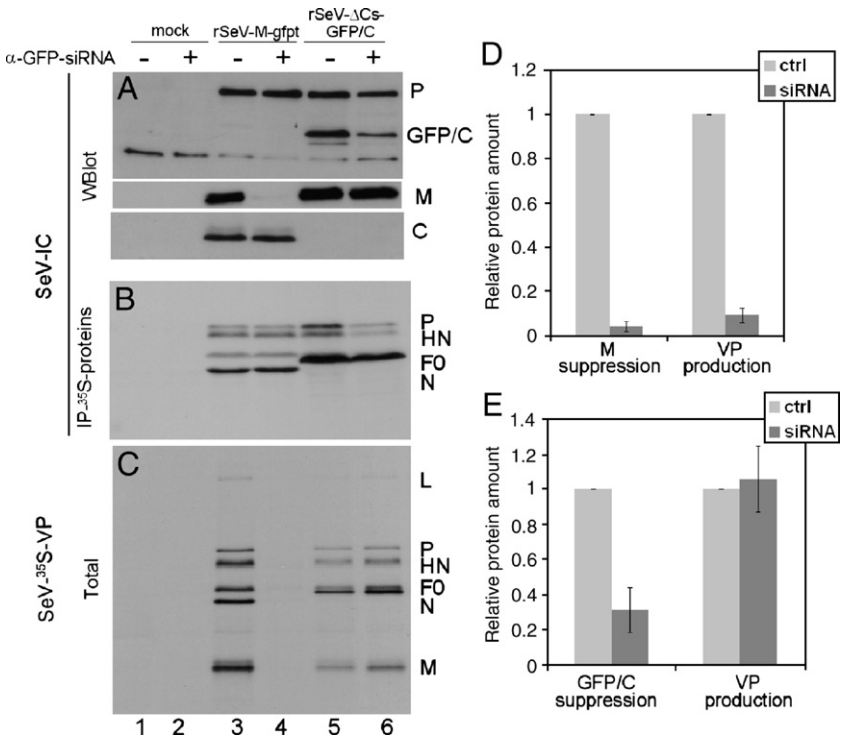

Fig. 7. Requirement for the C protein in SeV budding. A549-LV-siGFP or A549-LV-ctrl cells in 35 mm-diameter dishes were infected with rSeV-M-gfpt or rSeV-Δ4Cs-GFP/C (m.o.i. of 2), and radiolabeled from 16 h to 40 h pi. Culture medium and cells were harvested at 40 h pi. Cell extracts were prepared and the virus particles present in the supernatants were isolated as described in Materials and methods. Forty micrograms of the cell extracts were used for Western blotting analysis using α-GFP, α-SeV PCV and α-M antibodies (A) and the remaining fraction of cell extracts was used for immune precipitation using an anti-SeV rabbit serum (LIIIAb) (B). About 1/6th (adjusted to the total protein amount in the cell extracts) of the virus particles (C) were analyzed by SDS-PAGE. The radioactive gels (B and C) were treated for autoradiography. (D) M suppression: the bands of SeV-M in cell extracts were quantified and expressed relative to the values obtained in control cells normalized to 1. VP production: the bands of viral proteins in virus particles were quantified, summed up and expressed relative to the control conditions (no siRNA) normalized to 1. Standard deviations from the mean are indicated (4 experiments). (E) As in D except for GFP/C (4 experiments).

the involvement of cellular partners in SeV budding. The relevance of this interaction, reported to take place via a proposed YLDL domain, remains, however, to be shown. On the one hand, co-expression of M and wt Alix did not increase M shedding, and, on the other hand, of the two Alix deleted mutants that exhibited a negative effect on M shedding, one was shown not to interact with M. Furthermore, YLDL could not be replaced by other known late domains, so that it is not clear whether YLDL constitutes a newly described late domain, or whether the YLDL mutations annihilate M function in general, a fact that would not necessarily link this interaction to the MVB machinery recruitment. This latter possibility fits with our failure to show that M shedding was not affected by DN-VPS4A expression or Alix suppression (Fig. 5).

In the end, we have no clear explanation for the discrepancies between our conclusions and the one drawn mainly on the mechanism of SeV VLP production. It is possible that VLP production represents a more appropriate experimental system to unravel the details of virus particle formation. It is equally possible that complete virus particle formation in the course of an infection follows different paths. As for the C–Alix interaction, this may concern other cellular mechanisms in which both partners are known to be involved, like IFN interdiction for C or apoptosis triggering for Alix (Odorizzi, 2006). In that respect, it is relevant to mention that the deletion of the 10 C-ter amino acids of the C protein, known to break the C/Alix complex (Sakaguchi et al., 2005), destroyed the C ability to interdict with the IFN system (Dominique Garcin, not shown). As for the M–Alix interaction, it may be relevant that both partners are known to interact with actin in processes that are related to cytoskeleton remodeling and/or formation of the bud (Giuffre et al., 1982; Pan et al., 2006). Whether this represents a "ménage à trois" with a purpose different from the recruitment of the MVB machinery is open to question. At this point we acknowledge the need for further studies to be able to get a clearer picture of the SeV particle formation and of the respective role of the C–Alix or M–Alix interactions in this process.

Materials and methods

Cells

All cells were grown at 37 °C under 5% CO_2 atmosphere. HeLa, LLC-MK2 and A549 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS). FTGH, 293T (human renal epithelial cells expressing the simian virus 40 large T antigen) and Vero cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FCS. The preparation of

the A549-LV-siGFP cells is described in details elsewhere (Mottet-Osman et al., 2007). In brief, A549 cells were transduced with a lentiviral vector expressing siRNAs targeted to a defined sequence (gfpt) of the green fluorescent protein (GFP) gene and expressing the nerve growth factor receptor (NGFR) as described in (Wiznerowicz and Trono, 2003). The A549-LV-ctrl cell lines were transduced with a lentiviral vector expressing the NGFR alone. The transduced cells were selected on the basis of efficient NGFR surface expression. The FTGH-α-Alix-siRNA and 293T-α-Alix-siRNA cell lines were generated with the same technology. FTGH and 293T cells were transduced with a lentiviral vector expressing the GFP and siRNAs targeted to a defined sequence of the Alix gene (5′-GAGCTGTGTGTTGTTCAATT-3′). The FTGH-ctrl and 293T-ctrl cell lines were transduced with a lentiviral vector expressing the GFP alone. The transduced cells were selected on the basis of efficient GFP expression.

Antibodies

Antibodies used in this study include a rabbit serum raised against all the SeV proteins (LIIIAb), anti-SeV HN (a rabbit serum raised against SDS-denatured HN protein, α-HN_{SDS}), anti-SeV N (a rabbit serum raised against SDS-denatured N protein, α-N_{SDS}), anti-SeV M (MAb 383 obtained from Claes Örvell (Laboratory of Clinical Virology, Huddinge Hospital, Huddinge, Sweden), anti-SeV PCV (a rabbit serum raised against P, C and V proteins, α-PCV), anti-Influenza virus HA epitope MAb (16b12, Covance Research Products Inc.) and anti-GFP (a rabbit polyclonal serum raised against the full-length GFP protein, BD Living Colors Antibodies, Clontech Laboratories). The anti-Alix antibody was a gift from Gisou van der Goot (EPFL, Lausanne, Switzerland). The antibodies raised against SV5-HN, -NP and -M proteins were a gift from Randall RE (University of St. Andrews, Scotland, United Kingdom). Peroxidase-coupled secondary antibodies were from BioRad.

Plasmids

The plasmids encoding GFP-VPS4A and dominant negative mutants VPS4A-E228Q and VPS4A-K173Q were kindly provided by Robert A. Lamb (Northwestern University, Evanston, USA). The pEBS-HA-M plasmid was prepared from pEBS-GFP (Mottet-Osman et al., 2007) by replacement of the GFP gene with that of the HA tagged M (Mottet et al., 1996).

Viruses

Sendai virus was prepared and characterized as before (Roux and Holland, 1979). rSeV-M-gfpt is a recombinant SeV harboring in its M gene 5′ untranslated region a sequence derived from the GFP gene (5-AAGAACGGCAUCAAGGU-GAACUUAGC-3). Detailed description of the preparation of this rSeV is done in details elsewhere (Mottet-Osman et al., 2007). rSeV-Δ4C-GFP/C was constructed by Machiko Nishio while on sabbatical leave in Daniel Kolakofsky laboratory (University of Geneva, Switzerland). This rSeV does not express C proteins by introduction of 3 stop codons in the open reading frame of Y2, but expresses a GFP-fused C protein from a supplementary transcription unit inserted between the F and the M genes. SV5 (WR strain) was a kind gift from Machiko Nishio (Mie University Graduate School of Medicine, Japan) and was propagated on Vero cells.

Virus infections and radiolabeling

Infections with Sendai virus (SeV), its various recombinants (rSeV) or SV5 were performed at 33 °C. Virus stocks were adequately diluted (multiplicity of infection indicated in the figure legends) in DMEM without FCS and laid over the cells for 1 h. At the end of the infection period, the infectious mix was removed and replaced with fresh DMEM supplemented with 2% FCS. For radiolabeling, cells were incubated with 20 μCi of L-^{35}S-methionine and L-^{35}S-cysteine (Pro-mix-[^{35}S] in vitro cell labeling mix, Amersham Biosciences) in DMEM containing 1/10th the normal methionine and cysteine content plus 0.2% FCS, from 16 to 40 h pi. In the other cases, the medium was replaced at 16 h pi. by fresh medium containing 2% FCS. Culture medium and cells were harvested at the time indicated in the figure legends and analyzed as described below.

Transfections

HeLa cells in 35 mm-diameter dishes were transfected with 20 μM of anti-Alix siRNA (target sequence: 5′-AAGCCGC-TGGTGAAGTTCATC-3′, from Qiagen) using Oligofectamine (Invitrogen) according to the manufacturer's instructions, 48 h before SeV infection. 293T cells in 35 mm-diameter dishes were transfected with 2 μg of pVPS4A-wt, pVPS4A-E228Q or pVPS4A-K173Q using the FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions, 6 h before infection, or 16 h previous to pEBS-HA-M transfection.

Cellular extracts and immunoprecipitations

Transfected or infected cells were collected and disrupted in 300 μl of Lysing Buffer II (150 mM NaCl, 1% deoxycholate, 1% triton X-100, 0.1% sodium dodecyl sulfate, 10 mM Tris–HCl, pH 7.4) containing 1% aprotinin and 20 mM AEBSF as described before (Mottet et al., 1986). After 10 s of sonication (Branson Sonic Sonifer B-12, lowest speed), cell extracts were spun for 10 min at 12000 rpm in a microfuge. The supernatants were then processed for Western blotting analysis or IP. IP was performed as described before (Mottet et al., 1986). The total cellular extracts were first incubated with the specific antisera overnight at 4 °C, then with 50% suspension of protein A-sepharose 2 h at 4 °C to recover the immune complexes.

Virus particle or M-VLPs isolation

The virus particles or the M-VLPs were isolated from the clarified cell supernatants by centrifugation through a 25%

glycerol cushion (Beckman SW55 rotor, 2 h, 50,000 rpm, 4 °C) and directly resuspended in SDS sample buffer.

SDS-PAGE analyses, Western blotting, autoradiography and quantification

The total cellular extracts and the virus particles or the M-containing vesicles were analyzed by SDS-PAGE. After electrophoresis, the proteins were transferred using a semi-dry system onto polyvinylidene difluoride membranes (Millipore). Blots were then incubated with specific antibodies, followed by the appropriate horseradish peroxidase (HRP)-coupled secondary antibodies. Protein detection was performed by using the enhanced chemiluminescence system (Amersham Biosciences). The radiolabeled virus particle samples and the total IP samples were analyzed by SDS-PAGE and the gels, treated for enhanced fluorography (DMSO plus 5% PPO), were exposed to Hyperfilm MP (Amersham Biosciences). The films were scanned and the band signals were quantified with ImageQuant TL software (Amersham Biosciences).

Virus titration

Culture mediums from SeV-infected cells were collected and previously treated 30 min at 37 °C with 1.5 μg/ml of acetylated trypsin. SeV titers were determined by plaquing on LLC-MK2 cells under a 0.3% agarose overlay in the presence of 1.5 μg/ml of acetylated trypsin according to Sugita et al. (1974). SV5 titers were determined by plaquing on Vero cells under a 0.5% agar overlay.

Acknowledgments

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