Phenotypes of vesicular stomatitis virus mutants with mutations in the PSAP motif of the matrix protein

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Vesicular stomatitis virus (VSV) matrix protein (M) has a flexible amino-terminal part that recruits cellular partners. It contains a dynamin-binding site that is required for efficient virus assembly, and two motifs, 24PPPY27 and 37PSAP40, that constitute potential late domains. Late domains are present in proteins of several enveloped viruses and are involved in the ultimate step of the budding process (i.e. fission between viral and cellular membranes). In baby hamster kidney (BHK)-21 cells, it has been demonstrated that the 24PPPY27 motif binds the Nedd4 (neuronal precursor cell-expressed developmentally downregulated 4) E3 ubiquitin ligase for efficient virus budding and that the 37PSAP40 motif, although conserved among M proteins of vesiculoviruses, does not possess late-domain activity. In this study, we have re-examined the contribution of the PSAP motif to VSV budding. First, we demonstrate that VSV M indeed binds TSG101 [tumour susceptibility gene 101; a component of the ESCRT1 (endosomal sorting complex required for transport 1)] through its PSAP motif. Second, we analysed the phenotype of several recombinant mutants. We show that a double mutant with point mutations in both the PSAP and the PPPY motifs is impaired compared with a single mutant in the PPPY motif, indicating that the PSAP motif partially compensates for the lack of the PPPY motif. Mutants’ phenotypes depend on cell lines: in CERA (chicken embryo-related, Alger clone) cells, a recombinant virus with a single mutation in the PSAP motif was impaired compared with the wild type, and a mutant with a single mutation in the dynamin-binding motif was much less impaired in Vero cells than in BSR (clones of BHK-21) cells. These results have implications for the VSV budding pathway that will be discussed.

INTRODUCTION

Vesicular stomatitis virus (VSV) is the prototype rhabdovirus and has been used as a model for years to study many aspects of the virus life cycle. Its negative-strand RNA genome of 11 161 nt encodes successively nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (L). The N, P and L proteins are associated with the RNA molecule and compose the transcriptionally active nucleocapsid (NC). The NC is enveloped by a lipid bilayer derived from the host-cell plasma membrane during the budding process. G is a transmembrane glycoprotein that is involved in virus entry. Most of the M protein is located beneath the viral membrane and is bridging the NC and the lipid bilayer (Ge et al., 2010), but a significant M subpopulation might be located inside the NC helical coil (Barge et al., 1993; Libersou et al., 2010).

M is a multifunctional protein involved in virus assembly and budding. In relationship with this role, it has been shown that M interacts with both artificial and cellular membranes (Bergmann & Fusco, 1988; Chong & Rose, 1993; Luan & Glaser, 1994; Luan et al., 1995; Solon et al., 2005) and that it binds to the viral NC (Flood & Lyles, 1999; Lyles et al., 1996). It also self-associates into large multimers at physiological salt concentrations (Gaudin et al., 1995, 1997; McCreedy et al., 1990). As these interactions are supposed to be important features for both virus assembly and initiation of the budding process, the domains of M involved in the formation of all these complexes have been identified and characterized (Dancho et al., 2009; Gaudier et al., 2002; Graham et al., 2008).

Furthermore, VSV M has the ability to recruit cellular partners that assist virus assembly and budding. The first 10 aa of M constitute a dynamin-binding site. Disruption of this interaction affects the cellular localization of both G and N proteins and, as a consequence, virus assembly is impaired (Raux et al., 2010). The flexible amino-terminal part of M contains two motifs, 24PPPY27 and 37PSAP40, that constitute...
potential late domains (Craven et al., 1999). Late domains are found in proteins of many enveloped viruses and have the ability to recruit cellular partners that are involved in the ultimate step of the budding process (i.e. fission between viral and cellular membranes). Mutations in these motifs lead to phenotypes in which budding virions, although assembled normally, fail to be released and accumulate as tethered particles on cellular membranes (Demirov et al., 2002; Göttlinger et al., 1991; Jayakar et al., 2000). P(S/T)AP motifs recruit the protein tumour susceptibility gene 101 (TSG101) (Garrus et al., 2001), a component of ESCRT (endosomal sorting complex required for transport) complexes that play a key role in the biogenesis of multivesicular bodies (MVB). PPxY motifs have been shown to interact with WW domains of neuronal precursor cell-expressed developmentally downregulated 4 (Nedd4)-related E3 ubiquitin ligases (Harty et al., 1999; Timmins et al., 2003) that are supposed to be involved in cargo recruitment during MVB formation. Mutations in the PPPY motif of VSV M affect the efficiency of virus budding in baby hamster kidney (BHK)-21 cells (Jayakar et al., 2000). In striking contrast, mutations in the PSAP motif were without effect on virus assembly and budding in BHK-21 cells. Together with the fact that small interfering RNA-mediated depletion of cellular TSG101 was without effect on virus yield, this indicated that recruitment of TSG101 by M is dispensable (Irie & Harty, 2005; Irie et al., 2004).

The absence of a role for TSG101 raises some questions about the conservation of the PSAP motif in the M protein of the vesiculoviruses infecting mammals (VSV Indiana, VSV New Jersey, Piry virus, Isfahan virus, Cocal virus, Alagoas virus, Chandipura virus) (Brun et al., 1995; Marriott, 2005; Pauszek et al., 2008). In this study, we have thus examined the contribution of the PSAP motif to VSV. For this, using different cell lines we have compared the phenotype of recombinant VSV with mutations affecting M’s ability to bind to dynamin, Nedd4 and/or TSG101. We demonstrate that a double mutant with point mutations in both the PSAP and the PPPY motifs is much more impaired than a single mutant having only a mutation in the PPPY motif. This indicates that the PSAP motif partially compensates for the lack of a functional PPPY motif. Furthermore, in one cell line, a recombinant virus with a single mutation in the PSAP motif was impaired when compared with the wild type (WT). Implications concerning the budding pathway of VSV will be discussed.

RESULTS

VSV M protein interacts with both Nedd4 and TSG101

The flexible amino-terminal part of M contains two motifs, $24^{PPPYP}_{27}$ and $37^{PSAP}_{40}$ (Fig. 1a), that have been proposed to interact with Nedd4 and TSG101. The interaction of VSV M protein with both partners was validated using the yeast two-hybrid system (Fig. 1b).

![Fig. 1. (a) Structural organization of VSV M. The two late motifs ($24^{PPPYP}_{27}$ and $37^{PSAP}_{40}$) are indicated, as well as the residues (SSL) that have been demonstrated to be involved in dynamin binding (Raux et al., 2010). The flexible amino-terminal part of the protein is indicated in white, whereas the structured globular domain is indicated in grey. (b) Interaction of Nedd4 and TSG101 with WT and mutant M proteins M.Y27A and M.P40A in the two-hybrid system. The interactions between WT and mutant M proteins fused to the DNA-binding domain of LexA (LexBD) and cellular proteins (Nedd4 and TSG101) fused to the GAL4 activation domain were assessed by the appearance of blue colonies in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).](image)

![Fig. 2. Comparison of the morphology and the size of the plaques of WT VSV, VSVM.P40A, VSVM.Y27A, VSVM.L4A and VSVM.Y27A-P40A.](image)
The Nedd4 clone that we used was isolated during a two-hybrid screen, using the full-length VSV M protein as bait, against a nerve growth factor-induced PC12 cell (rat adrenal pheochromocytoma cell line) cDNA library (Raux et al., 2010). The human TSG101 clone was a gift of Winfried Weissenhorn.

As expected, disruption of the \(^{24}\text{PPPY}^{27}\) motif by replacing tyrosine in position 27 by an alanine (mutant M.Y27A) abolished the interaction between M and Nedd4 (Fig. 1b) in agreement with previous results of Jayakar et al. (2000).

Similarly, disruption of the \(^{37}\text{PSAP}^{40}\) motif by replacing proline 40 by an alanine (mutant M.P40A) also abolished the interaction between M and TSG101 (Fig. 1b). This demonstrated that the PSAP motif in the context of the VSV M sequence is able to recruit TSG101, although this interaction does not seem to play a role in the budding process (Irie et al., 2004).

**Recovery and characterization of recombinant VSV containing M protein mutations**

The effects of mutations in the PPXY and PSAP motifs in the context of the viral infection were also investigated. For this, the genes encoding the WT M protein (Orsay strain), the PPXY mutant (referred to as M.Y27A), the PSAP mutant (referred to as M.P40A) and the double mutant (referred to as M.Y27A-P40A) were subcloned into full-length VSV cDNA. All of the corresponding viruses were recovered and could be passaged in cell culture. Therefore, the mutations did not prevent the release and the spread of the virus.

The plaque size of the mutant containing a single mutation P40A was the same as that of WT virus, whereas the plaques formed by the mutants containing the Y27A mutation were smaller (Fig. 2). It is noteworthy that the plaques formed by the double mutant M.Y27A-P40A were smaller than those formed by mutant M.Y27A. Thus, in the context of mutant M.Y27A, the mutant M.P40A has a phenotype. We also characterized the plaque morphology of mutant M.L4A, for which the M protein is unable to bind dynamin (Raux et al., 2010). As already described, it was very different from that of the WT virus and of the other mutants. The plaques appeared fuzzy as if infection-induced cell lysis was incomplete.

We analysed and compared the production of recombinant WT virus and mutants M.Y27A, M.P40A, M.Y27A-P40A and M.L4A, in BSR cells. The virus released in the culture medium after 6 h was purified and analysed by SDS-PAGE (Fig. 3a). In parallel, the amount of viral proteins present in infected cell extracts was quantified by Western blot analysis (Fig. 3b). Similar amounts of M, N, P and G proteins (L was not detected by the anti-VSV serum) were found in cells infected either by WT VSV or by the mutants. In contrast, the amount of mutant viruses M.Y27A, M.Y27A-P40A and mutant M.L4A released in the culture medium was strongly reduced. The amount of
mutant viruses M.Y27A and M.L4A was reduced to about 5% of that of WT virus (Fig. 3a). Proteins of mutant M.Y27A-P40A were not detected and thus could not be quantified. The amount of mutant virus M.P40A was only slightly reduced, corresponding to about 70% of WT virus.

One-step (m.o.i. = 3) growth curves of the mutants were compared with those of the WT in BSR cells (Fig. 3c). The growth curve of mutant M.P40A was similar to that of the WT. For the other three mutants, a short delay in virus release was observed at early time points. After 6 h of infection, the titre was reduced by a factor of about 40, 60 and 360 for mutants M.L4A, M.Y27A and M.Y27A-P40A, respectively (Table 1, line BSR).

**Electron microscopy of infected BSR cells and purified virus particles**

Thin sections of infected BSR cells were examined by electron microscopy (Fig. 4). For both WT and M.P40A, electron micrographs showed a few bullet-shaped virions in the process of budding at the cell surface. In contrast, for M.L4A virtually no virus particles were observed. For mutants M.Y27A and M.Y27A-P40A, huge accumulations of virions in the process of budding were observed at the cell surface (Fig. 4).

We then used negative staining to investigate the morphology of purified virus particles (Fig. 5). The morphology of M.P40A and M.L4A could not be distinguished from that of WT VSV. Observation of mutant M.Y27A revealed some particles with aberrant branched morphology, but many virions still kept their typical bullet shape. For the double mutant M.Y27A-P40A, only a few virus particles seemed to have kept the WT morphology and many virions exhibited branched or even very elongated structures reminiscent of those of filoviruses.

**Characterization of mutants produced in other cell lines**

Taken together, the results presented above with mutants M.P40A, M.L4A and M.Y27A were consistent with previous data: (i) mutant MP40A, of which the M protein is unable to bind TSG101, is not affected; (ii) mutant M.L4A is affected at a stage of virus assembly; and (iii) mutant M.Y27A is affected at a late stage of the budding process (i.e. fission between the viral and cellular membranes).

The data obtained with double mutant M.Y27A-P40A were not expected. They reveal that the mutation P40A has a marked phenotype in the context of the mutant M.Y27A. This suggested that M binding to TSG101 could somehow compensate for the lack of interaction with Ned4. We thus hypothesized that the requirement of TSG101 for efficient viral budding may vary depending on the cell line and that in some cell lines the mutation P40A might produce a phenotype even in the absence of mutation Y27A.

As it has been shown that although VSV budding occurs in general at the cytoplasmic membrane, in primary embryo chick fibroblasts, budding particles can also be observed in intracellular vacuoles or vesicles (Hackett et al., 1968), we decided to investigate the assembly and budding of both WT VSV and mutants in fibroblastic CERA (chicken embryorelated, Alger clone) cells, and also in epithelial Vero cells.

Experiments were performed as those on BSR cells and we analysed the production of recombinant WT virus and the different mutants in both CERA and Vero cells.

**Table 1.** Virus titres corresponding to those determined for recombinant WT virus and mutants at 6 h p.i. in Fig. 3(c) (for BSR cells) and Fig. 6(c) (for CERA and Vero cells)

<table>
<thead>
<tr>
<th>Cells/characteristic</th>
<th>WT</th>
<th>Mutants</th>
<th>M.P40A</th>
<th>M.Y27A</th>
<th>M.L4A</th>
<th>M.Y27A-P40A</th>
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<td>BSR</td>
<td></td>
<td></td>
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<tr>
<td>Titre (p.f.u. ml⁻¹)</td>
<td>3.3 × 10⁸</td>
<td>2.5 × 10⁸</td>
<td>5.7 × 10⁶</td>
<td>8.8 × 10⁶</td>
<td>9.2 × 10⁵</td>
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<td>WT titre/titre</td>
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<td>37</td>
<td>360</td>
<td></td>
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<td>Particle infectivity</td>
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<td>0.38</td>
<td>0.41</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>CERA</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Titre (p.f.u. ml⁻¹)</td>
<td>7.2 × 10⁷</td>
<td>2.7 × 10⁷</td>
<td>1.5 × 10⁶</td>
<td>3.1 × 10⁶</td>
<td>3.3 × 10⁵</td>
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</tr>
<tr>
<td>WT titre/titre</td>
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<td>ND</td>
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<td>ND</td>
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</tr>
<tr>
<td>Vero</td>
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<td>4.9 × 10⁶</td>
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<td>5.8 × 10⁵</td>
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<td>Particle infectivity</td>
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<td>ND</td>
<td>0.53</td>
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Particle infectivity (normalized to 1 for WT virus) was determined using the percentage yield of proteins N/P and M indicated in Figs 3(a) and 6(a) by the following formula: particle infectivity = [(percentage yield of N/P + percentage yield of M)/2] × (WT titre/titre)/100. ND, Not determined.
The virus released in the culture medium after 6 h was purified and analysed by SDS-PAGE (Fig. 6a) and the amount of viral proteins present in infected-cell extracts was quantified by Western blot analysis (Fig. 6b). In both cell lines, as in BSR cells, similar amounts of M, N, P and G proteins were found in cells infected either by WT VSV or by the mutants. Two main differences were observed when the results were compared with those obtained in BSR cells. First, in CERA cells the amount of mutant virus M.P40A released in the culture medium was reduced to 50% of that of WT. Second, the mutant virus M.L4A was much less affected in Vero cells than in BSR cells as the amount of virus released in the culture medium was only reduced to about 70% of that of WT (instead of about 6% in BSR cells). For the other combinations of mutants and cells, the results were rather similar to those obtained in BSR cells.

One-step (m.o.i. = 3) growth curves of the mutants were also compared with those of WT in Vero and CERA cells (Fig. 6c). In CERA cells, after 6 h of infection, the titre was reduced by a factor of about 25, 50 and 220 for mutants M.L4A, M.Y27A and M.Y27A-P40A, respectively (Table 1, line CERA). These titre reductions were very similar to those obtained in BSR cells. Here again, mutant M.P40A appeared to be markedly more affected than in BSR cells with a titre reduction by a factor of 2.7.

In Vero cells, mutants were less affected than in BSR or CERA cells. This was particularly remarkable for mutant M.L4A for which, at 6 h post-infection (p.i.), the titre was only reduced by a factor of 2.5 (versus about 25 and 40 in CERA and BSR cells, respectively) (Table 1, line Vero).

Thus, the growth curves data were globally consistent with the data on the amount of physical particles released in the culture medium.

We also performed electron microscopy on infected CERA and Vero cells. No obvious difference with infected BSR cells was detected. In both cell types (CERA and Vero), neo-synthesized virions were budding at the plasma membrane. Furthermore, although mutant M.L4A was markedly less affected in Vero cells, very few virus particles were observed in the process of budding (as in the other cell types).

**DISCUSSION**

In this study, we have investigated the role of the interaction between VSV M protein and three cellular partners that bind its flexible amino-terminal segment. Two of these partners, Nedd 4 and dynamin, have already been demonstrated to be required for efficient fission at the end of the budding process (Harty et al., 1999, 2001; Jayakar et al., 2000) and for virus assembly (Raux et al., 2010), respectively. The third partner, TSG101, binds the PSAP motif of M. Nevertheless, it has been shown that interaction between M and TSG101 is not crucial for efficient VSV budding. Particularly, it has been reported that, in BHK-21 cells, the PSAP motif of VSV M does not possess late-domain activity similar to that of the PPPY motif and that the infectious cycle of mutants for which the PSAP motif was disrupted does not seem to be affected (Irie et al., 2004). Thus, the fact that the PSAP motif is

![Fig. 4. Electron micrographs of BSR cells infected by VSV WT, VSV.M.P40A, VSV.M.L4A, VSV.M.Y27A and VSV.M.Y27A-P40A recombinant viruses at 6 h p.i. Bars, 1 μm. Virions in the process of budding at the plasma membrane of cells infected by the different viruses are indicated by arrows.](http://vir.sgmjournals.org)
conserved among many vesiculoviruses infecting mammals was intriguing.

Our first observation is that mutation M.P40A, which renders M unable to bind TSG101, has indeed a phenotype in the context of mutation M.Y27A. Double mutant M.Y27A-P40A is always much more affected than single mutant M.Y27A: whatever the cell line used, the yield of infectious particle was systematically decreased by a factor of 5–10 (Table 1). As for the single mutant, the double mutant was affected at a late stage of the virus life cycle, consistent with the idea that somehow the PSAP motif can partially compensate for the absence of the PPPY motif.

We also show that, in CERA cells, mutant M.P40A is markedly affected when compared with the WT. This indicates that, in CERA cells, the VSV PSAP motif has some late-domain activity. The phenotype of M.P40A in CERA cells exemplifies the fact that interaction with a cellular partner can be more specifically required in some cell types. Consistent with this view, the phenotype of mutant M.L4A is much less pronounced in Vero cells than in BSR or CERA cells, indicating that the level of requirement of the interaction between M and dynamin varies from one cell type to another.

In BSR cells, virus particles produced after cell infection with mutants M.Y27A and M.Y27A-P40A often have a branched and/or filamentous morphology, a feature that is exacerbated with the double mutant. It seems that these aberrant particles are less infectious as the ratio of physical to infectious particle is lower for mutant M.Y27A (Table 1). It should be noted that a similar decrease of infectivity of physical particles is also observed with mutant M.L4A. This suggests that some defect, so far undetected, might be associated with the virions produced in the absence of interaction between M and dynamin.

Taken together, our data indicate that different cellular actors can mediate the fission of VSV in an independent manner. In BSR and Vero cells, the preferential pathway requires the PPPY motif and interaction with Nedd4, whereas in CERA cells the PSAP motif and interaction with TSG101 are also contributing significantly to the process.

These data also illustrate the more general notion that viruses have evolved to exhibit more than one solution to fulfill their multiplication cycle. This notion is particularly relevant for vesiculoviruses, which are often transmitted by insect vectors. Indeed, for efficient budding from insect cells, it is highly probable that the M protein interacts with TSG101- and Nedd4-analogous insect proteins.

Finally, it is worth noting that the fixed laboratory VSV strains are the result of a long adaptation process due to several passages in cell culture. This adaptation may have selected a budding pathway that may not be the major (or the only) one in the natural life of the virus.

**METHODS**

**Cells and antibodies.** BSR, clones of BHK-21 (baby hamster kidney) cells, and Vero 76, clones of Vero (African green monkey kidney) cells, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10 % FCS. CERA (chicken embryo-related, Alger clone) cells were grown in modified Eagle’s medium supplemented with 10 % FCS.

**Recovery of recombinant virus.** Plasmids pVSV-FL(+) expressing the 11 161 nt positive-strand full-length VSV RNA sequence, pBS-N, pBS-P, and pBS-L, respectively encoding N, P and L proteins, were kindly provided by John K. Rose (Yale University, New Haven, CT, USA). The WT or mutant M genes of the VSV Indiana serotype (Orsay strain) were inserted into the original full-length genomic plasmid pVSV-FL(+) (Lawson et al., 1995) using two unique sites of pVSFPFL(+), XhoI in the C-terminal region of the phosphoprotein (P).
gene and Mlu in the 5′ non-coding sequence of the glycoprotein (G)
gene, after removal of the corresponding VSV Indiana Mudd Summers
M gene. Recombinant VSV was recovered as described by Schnell et al.
(1996). The plasmids were transfected into the cells with Fugene 6
(Roche Diagnostics) in the presence of 10 μg AraC ml−1 (Sigma).
Working stocks of recombinant virus were prepared as follows.
Monolayers of BSR cells were infected at an m.o.i. of 0.1. After 18–
24 h, supernatants were collected and cellular debris was removed by
low-speed centrifugation (2500 g for 15 min). Initial titres were determined
by standard plaque assay onto BSR cells and the M gene was sequenced.

**Plasmid constructs used in the yeast two-hybrid assay.**
Expression constructs for TSG101 were derived from full-length
cDNA kindly provided by Winfried Weissenhorn (Unit of Virus Host
Cell Interactions, UMR5233 UJF-EMBL-CNRS, Grenoble, France).
The Nedd4 clone that we used was isolated during a previous

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**Fig. 6.** Characterization of the production of recombinant WT virus and mutants in CERA cells (left panels) and in Vero cells
(right panels). (a) Protein profiles for VSV WT, VSVM.P40A, VSV.M.Y27A, VSVML4A and VSVM.Y27A-P40A. Virions were
harvested from the supernatant of infected cells at 6 h p.i. and virion proteins were analysed by SDS-PAGE and Coomassie
blue staining as described in Fig. 3(a). (b) Lysates of the infected cells were analysed by Western blotting as described in Fig.
3(b). (c) Growth kinetics of VSV WT (●), VSVM.P40A recombinant virus (■), VSVM.Y27A recombinant virus (●), VSVML4A recombinant virus (▲) and VSVM.Y27A-P40A recombinant virus (×). The cells were infected at an m.o.i. of 3 and samples were
harvested for titration at the indicated times p.i. Virus titres represent means from at least three independent experiments.

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two-hybrid screen (Raux et al., 2010). cDNAs encoding either TSG101 or Nedd4 were fused to the sequence encoding the GAL4 activation domain and cloned into pGAD-GE (derived from pGAD-GH; Clontech). cDNAs encoding VSV M (Indiana, Orsay strain) and VSV M mutants were fused to the sequence encoding the DNA-binding domain of LexA and cloned into pLex (Clontech). The proper framing and accuracy of the sequences of all DNA constructs were confirmed by DNA sequencing.

**Yeast two-hybrid assay.** VSV M (bait), fused to the DNA-binding domain of LexA, was used to test the interaction with Nedd4 or TSG101 (preys) which were fused to the sequence encoding the GAL4 activation domain. The yeast L40 strain containing the reporter gene lacZ was transformed with the bait plasmid pLex-M and the prey plasmid pGAD-GE-Nedd4 or pGAD-GE-TSG101 by using a lithium acetate protocol. Double transformants were grown on plates containing medium lacking Trp and Leu (Trp- Leu-) to select for the presence of both the bait and the prey. Positive clones were then assayed for β-galactosidase activity. Interaction between LexA binding domain-M and GAL4 activation domain-cellular proteins conferred on L40 the ability to produce β-galactosidase activity, by reconstitution of transcription factor gene lacZ.

**Virus titration.** Virus titration was performed as follows. BSR cells on glass slides were infected by VSV at an m.o.i. between 0.3 and 0.9 (the virus titres had been previously roughly determined by standard plaque assays into BSR cells). Nuclei were stained using DAPI, allowing the number of cells to be counted, and the number of infected cells was determined by immunofluorescence with the rabbit anti- VSV polyclonal antibody (made in house).

The probability, \( p(n) \), for a cell to be infected by exactly \( n \) virus particles at an m.o.i. equal to \( i \) is given by the Poisson distribution (Grigorov et al., 2011) and thus \( p(n)=e^{-i}i^n/n! \). Consequently, the probability that a cell is not infected is \( p(0)=e^{-i} \). \( p(0) \) can be determined experimentally by counting the total number of cells and the number of non-infected cells. The m.o.i., which is equal to \(-\ln[p(0)]\), can then be calculated and the virus titres can be then determined. In our hands, this titration method appeared to be much more reproducible than the standard plaque assay (see also Grigorov et al., 2011).

**Quantification of virus particles present in cell supernatant.** Monolayers of cells (either BSR, CERA or Vero) were grown to confluence in 150 mm diameter dishes and infected at an m.o.i. of 3 for 6 h. Supernatants of infected cells were first clarified at 2400 rpm for 30 min. Then they were loaded onto a cushion of 25% glycerol/TNE buffer (10 mM Tris/HCl pH 7.5, 50 mM NaCl, 1 mM EDTA) and centrifuged for 1 h at 25 000 r.p.m. in a SW 32 Ti Beckman rotor. The pellets were resuspended in Laemmli buffer (Laemmli, 1970) for SDS-PAGE analysis.

**Electron microscopy.** For transmission electron microscopy, BSR cells were grown to confluence in 100 mm diameter Petri dishes and infected at an m.o.i. of 3. At 5 h p.i., the cells were fixed for 1 h at room temperature with 2% glutaraldehyde in 100 mM cacodylate buffer pH 7.4 (Sigma). Cells were washed with 0.1 M sucrose (Sigma) in 0.1 M cacodylate buffer (pH 7.4). Cells were then post-fixed for 1 h at room temperature with 1% aqueous osmium tetroxide (OsO4) and 1.5% potassium ferrocyanide (Sigma) in 0.1 M cacodylate buffer (pH 7.4) and stained with 2% uranyl acetate in water. The cells were then dehydrated in increasing concentrations of ethanol and embedded by Epon with 2,4,6-tris(dimethylaminomethyl)-phenol (DMPS30; Delta Microscopies). Polymerization was carried out for 48 h at 60 °C. Ultra-thin sections of Epon-embedded material were collected on copper palladium grids (200 mesh) and stored until use. These sections were stained with lead citrate (Delta Microscopies).

Sections were examined with a Zeiss EM902 electron microscope operated at 80 kV, and images were acquired with a charge-coupled device camera (Megaview III) and analysed with ITEM software [Éloïse, France; MIMA2 platform (INRA-CAIR)].

For negative staining, virus particles were purified from the culture supernatant 15 h after infection at an m.o.i. of 3. Cell debris was eliminated by centrifugation for 30 min at 3500 r.p.m. in a rotor (model JA14; Beckman Coulter) at 4 °C. The virus was then recovered as a pellet by centrifugation for 3 h at 14 000 r.p.m. in the JA14 rotor at 4 °C and resuspended in TD buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4 and 25 mM Tris/HCl, pH 7.5). Samples were adsorbed onto airglow discharge carbon-coated grids and stained with sodium phosphotungstic acid. Images were recorded in an electron microscope (model CM12; Philips) operated at 120 kV, with a nominal magnification of \( \times 31 000 \).

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