Rotavirus NSP5 orchestrates recruitment of viroplasmic proteins

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INTRODUCTION

Rotaviruses (RV), members of the family Reoviridae, are non-enveloped viruses with a segmented double-stranded RNA (dsRNA) genome. They infect a number of different mammalian species (including humans) and birds provoking diarrhoea (Estes & Kapikian, 2007). The infective virion is a triple-layered particle (TLP) with an internal core containing the viral genome (11 dsRNA segments), the RNA-dependent RNA polymerase VP1 and the RNA capping enzyme VP3, surrounded by an inner-most layer formed by pentamers of VP2. A second middle layer covering the core consists of trimers of VP6 giving rise to an intermediate double-layered particle (DLP), which upon acquiring the third outer-most layer of VP7 and VP4 forms the fully assembled viral particle (TLP) (Estes & Kapikian, 2007).

Each of the 11 genome segments encodes a single protein with the exception of segment 11, which in some strains has two open-reading frames (ORFs), one for NSP5 and the other for NSP6. Therefore, the infective particle encodes overall six structural (VP1, VP2, VP3, VP4, VP6 and VP7) and five or six non-structural (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) (Estes & Kapikian, 2007) proteins.

Early after infection (2–3 h) discrete structures known as viroplasms appear in the cytoplasm. They were shown to contain the structural proteins VP1, VP2, VP3 and VP6 and the non-structural ones NSP2 and NSP5 (Estes & Kapikian, 2007; Silvestri et al., 2004). Based on this protein composition and on electron microscopy analysis showing newly made cores and DLPs within them, viroplasms are considered the sites of viral genome replication and of the initial steps of virus morphogenesis (Altenburg et al., 1980; Esparza et al., 1980; Petrie et al., 1984). In addition, there is evidence that viroplasms are also the sites of plus-strand RNAs transcription (Silvestri et al., 2004). The mechanism of viroplasm assembly, however, is still unknown. The current and controversial hypothesis is that the incoming DLPs act as focal points around which accumulate the newly synthesized viral proteins to form viroplasms (Silvestri et al., 2004).

NSP2 and NSP5, the only non-structural proteins found in viroplasms, were shown to be required essentially for the formation of these structures and for viral infection (Camagnella et al., 2005; Lopez et al., 2005; Silvestri et al., 2004; Vascotto et al., 2004). While for NSP2 several roles
have been proposed, for example as a molecular motor for genome replication and packaging, the role of NSP5 is rather obscure (Arnoldi & Burrone, 2009). It has been demonstrated that NSP5 is able to strongly interact with at least two other viral proteins, namely NSP2 and VP1, both in virus-infected cells and when co-expressed with either of them in the absence of other viral proteins (Afrikanova et al., 1998; Arnoldi et al., 2007). During viral infection NSP5 is subjected to a complex phosphorylation process leading to the appearance of several isoforms, of which the most abundant has an apparent molecular mass of 28 kDa, followed by one of 26 kDa and by others ranging from 32 to 34 kDa (Afrikanova et al., 1996). When NSP5 is expressed alone it accumulates in the cytoplasm with a homogeneous distribution mostly as the isoform of 26 kDa (with very low phosphorylation level or non-phosphorylated) (Afrikanova et al., 1996). Interestingly, when NSP5 is co-expressed with NSP2 in uninfected cells, the two proteins interact and NSP5 becomes hyperphosphorylated producing isoforms of apparent higher molecular mass (Afrikanova et al., 1998). A second consequence of the direct interaction with NSP2 is its relocalization into discrete structures morphologically resembling viroplasms and named VLS (viroplasm-like structures) (Fabbretti et al., 1999). This property most likely depends on an NSP5 conformational change specifically induced by the interaction with NSP2, since the interaction with the viral polymerase VP1 does not induce VLS formation (Arnoldi et al., 2007).

In contrast to NSP2 and NSP5, the four structural proteins VP1, VP2, VP3 and VP6 appear not to be essential for formation of viroplasms (Ayala-Breton et al., 2009), despite the fact that they accumulate within viroplasms and are required for the early stages of virus morphogenesis (Estes & Kapikian, 2007).

In this study, we characterize the property of VLS to recruit all viroplasmic proteins and show the key role of NSP5 as the sole protein required for VLS formation.

RESULTS

VP2 induces VLS

VLS, which were initially described as structures induced by co-expression of NSP5 and NSP2, greatly resemble viroplasms (Supplementary Fig. S1a, b, available in JGV Online). Co-expression of NSP5 with VP2, however, also generates VLS. As shown in Fig. 1(a), immunofluorescence with anti-NSP5 and anti-VP2 antibodies of co-transfected cells revealed the presence of structures similar to those formed by NSP5 and NSP2, although less uniformly distributed. As in the case of NSP2, also VP2 was recruited into VLS. Recruitment of VP2 was easily detectable when the amount of the plasmid encoding VP2 was reduced (to 1/5th–1/10th) in relation to that of NSP5. Based on morphological similarities of VLS induced by NSP2 or VP2 and to distinguish between them, they were named VLS-NSP2i (NSP2 induced) and VLS-VP2i (VP2 induced). Formation of VLS and recruitment of VP2 was further confirmed by using the embryonic African green monkey kidney (MA104) stable cell line expressing the fusion protein NSP5–EGFP (Fig. 1b), with EGFP at the C terminus (Eichwald et al., 2004b). In these cells, fluorescent NSP5–EGFP becomes concentrated in viroplasms following rotavirus infection (Supplementary Fig. S1c, available in JGV Online). Upon transfection of NSP5 and VP2 encoding plasmids, formation of VLS-VP2i was observed both by autofluorescence of NSP5–EGFP and by immunofluorescence with anti-NSP5 antibodies, which, as expected, showed complete co-localization (Fig. 1b, upper panel). VP2 was found co-localized with NSP5–EGFP in these structures (Fig. 1b, lower panel). In this cell line, co-transfection of wild-type (wt) NSP5 is required to visualize VP2 recruitment into VLS (Supplementary Fig. S2, available in JGV Online), a characteristic also observed when co-expressing NSP2 (data not shown). This most likely reflects the requirement of an NSP5 expression level above a threshold.

The two types of VLS are very similar and seem to reflect true viroplasms. Indeed, when both NSP2 and VP2 were co-expressed with wt NSP5 in the NSP5–EGFP cell line, VLS containing all three components were observed, indicating that a macromolecular complex with all three proteins contributes to viroplasm formation (Fig. 1c). This is further confirmed by the analysis of the Z-series projections of cells co-expressing the same three proteins. A single projection of a rendered 3D reconstruction is shown as Supplementary Fig. S3 (available in JGV Online). As mentioned above, the need for wt NSP5 was probably required to accomplish an adequate ratio between the three viral proteins rather than to overcome malfunction of NSP5–EGFP. Indeed, MA104 cells transiently co-transfected with NSP5–EGFP and VP2, or NSP5–EGFP and NSP2, showed assembly of VLS with recruitment of VP2 or NSP2, respectively (Supplementary Fig. S4, available in JGV Online).

A direct interaction between NSP5 and VP2, which would be expected in view of the formation of VLS and VP2 recruitment, was elusive. Attempts to co-immunoprecipitate VP2 with anti-NSP5 antibodies or vice-versa failed, even when performed with extracts derived from cells treated in vivo with the bifunctional cross-linking reagent DSP (Afrikanova et al., 1998; Arnoldi et al., 2007; data not shown). An NSP5–VP2 interaction, however, has been previously reported from extracts of virus-infected cells using a monoclonal anti-NSP5 antibody (Berois et al., 2003).

VP2 induces phosphorylation of NSP5

We have recently reported that VP2 is able to induce a strong NSP5 hyperphosphorylation in the absence of other viral proteins (Arnoldi et al., 2007). Western blots...
experiments on extracts of cells co-expressing VP2 and NSP5 showed a remarkable increase of NSP5 hyperphosphorylation, represented by the emergence of the isoforms resembling those found in virus-infected cells, as opposed to the main isoform of 26 kDa of NSP5 expressed alone (Fig. 2a). Treatment with lambda-phosphatase confirmed that the effect of VP2 on NSP5 was due to increased hyperphosphorylation (Fig. 2b).

The mechanism by which VP2 increases NSP5 hyperphosphorylation remains obscure. We have previously demonstrated that protein kinase CK1α is involved in the initial steps required for the activation of the hyperphosphorylation cascade (Eichwald et al., 2004a). Experiments of RNA interference against CK1α in virus-infected cells have shown that despite impairment of NSP5 phosphorylation, viroplasm formation was not affected. Yet, morphological changes of viroplasms were observed (Campagna et al., 2007). Based on these results, we analysed VP2-induced NSP5 hyperphosphorylation upon silencing of CK1α. We observed that CK1α silencing had no effect on NSP5 hyperphosphorylation and VLS formation (data not shown).

VLS formation and NSP5 phosphorylation
Since VP2 and NSP2 induce both VLS formation and NSP5 hyperphosphorylation, we investigated whether there was a correlation between the two events. For this purpose we tested the formation of VLS in four different conditions, in which NSP5 hyperphosphorylation was partially or highly impaired: (i) co-expression of NSP5 and VP2 or NSP2 with the catalytic subunit of the cellular phosphatase PP2A (an

![Fig. 1. VLS assembly. Confocal immunofluorescence of (a) MA104 cells transfected with NSP5 (red) and VP2 (green). (b) NSP5-EGFP/MA104 cells transfected with NSP5 and VP2. Upper panel, anti-NSP5 (red); lower panel, anti-VP2 (red). (c) NSP5-EGFP/MA104 cells transfected with NSP5, VP2 (red) and NSP2 (blue). In (b and c) NSP5-EGFP is shown in green. Single optical sections are shown.](image)

![Fig. 2. VP2 induces NSP5 hyperphosphorylation. (a) Western blot of extracts of cells infected or co-transfected with the indicated genes. The upper and lower parts of the blots were reacted with specific anti-VP2 or anti-NSP5 sera, respectively. (b) Western blot of extracts of cells transfected with the indicated genes and treated with λ-phosphatase (λPPase).](image)
ubiquitous and conserved serine/threonine phosphatase with broad substrate specificity) produced a significant decrease of NSP5 hyperphosphorylation (Fig. 3a), but did not compromise formation of VLS (Fig. 3b); (ii) co-expression of NSP5 with either of the two VLS inducers (VP2 and NSP2) and VP1 (as an SV5 N-terminally tagged version) strongly hampered the effect of NSP5 hyperphosphorylation (Fig. 4a, lanes 4, 7 and Arnoldi et al., 2007), while the capacity to form VLS remained unaltered (Fig. 4b). It is possible that the inhibitory effect of VP1 on NSP5 phosphorylation is the consequence of their interaction, sequestering NSP5 as a substrate. Interestingly, in both VLS-VP2i and VLS-NSP2i, VP1 was found to be entirely recruited in contrast with its homogeneous distribution in the absence of VLS (i.e. when co-expressed with NSP5 or NSP2 or VP2, Fig. 4b); (iii) two NSP5 phosphorylation mutants NSP5-Ser67Ala (S67A) and NSP5-Ser63,65,67Ala (NSP5a) (Eichwald et al., 2004a), which showed impaired phosphorylation (altered ratio of 26/28 kDa isoforms) upon co-expression with VP2 or NSP2 (Fig. 5a), were still able to form VLS efficiently (Fig. 5b); (iv) since the effect of VP2 or NSP2 on NSP5 hyperphosphorylation depends on the relative amount of the inducer with respect to NSP5, co-transfection of plasmids at ratios not leading to increased NSP5 hyperphosphorylation (1/10, VLS inducer/NSP5; Fig. 6a) still allowed VLS formation (Fig. 6b).

In conclusion, NSP5 phosphorylation appears not to be involved in VLS formation.

From VLS to viroplasms

Although viroplasms are well-defined morphological entities containing six protein components of viral origin (VP1, VP2, VP3, VP6, NSP2 and NSP5), a detailed knowledge of the requirements for their assembly is still missing. VLS are the closest structures so far defined that contain viroplasmic protein components. With the aim of understanding the mechanism of viroplasm assembly, we co-expressed all viroplasmic proteins in different combinations and analysed their cellular localization by immunofluorescence using specific antibodies. As already shown in Fig. 1(c), in VLS formed by co-expression of NSP5, NSP2 and VP2 all three components showed clear co-localization, as long as they were expressed at defined ratios. Furthermore, VP1 was also recruited into VLS regardless of whether they were induced by VP2 or NSP2, co-localizing with NSP5 and VP2 or NSP2 and NSP2, respectively (Fig. 7a; see also Fig. 4b and Arnoldi et al., 2007). Similarly, co-expression of NSP5 and VP2 with the middle layer protein VP6 showed total recruitment of VP6 into VLS-VP2i (Fig. 7b, upper panel). In contrast, VP6 was not recruited into VLS-NSP2i (Fig. 7b, lower panel), suggesting a relevant role of the well-defined VP2–VP6 interaction for...
VP6 localization. Indeed, in the absence of VP2, conditions under which there are no VLS and NSP5 is diffuse, VP6 was found forming tubular structures as it does when expressed alone. When expressed with VP2, however, a punctuated distribution of VP6, clearly distinct from VLS, was observed (Supplementary Fig. S5, available in JGV Online).

A further step towards the formation of more complex VLS consisted in the co-expression of all six viroplasmic proteins NSP2, NSP5, VP1, VP2, VP3 and VP6. Under these conditions, unambiguous assembly of VLS with the concomitant recruitment of each component was observed (with the exception of VP3, because of the lack of a suitable antibody) (Fig. 8a). In these experiments, once again, the key role of NSP5 in VLS formation became evident when co-transfections of all viroplasmic components with the sole exception of NSP5 showed lack of VLS formation (Fig. 8b).

Taken together these results suggest that VLS could be considered as intermediates of viroplasm maturation with NSP5 playing a central role as it does in the assembly of true viroplasms.

DISCUSSION

The molecular mechanism underlying the assembly of viroplasms in RV-infected cells has not yet been elucidated. An essential role of the two non-structural proteins NSP2

Fig. 4. VP1 downregulates NSP5 phosphorylation without affecting VLS formation. (a) Western blot of extracts of cells transfected with the indicated genes. The upper, middle and lower parts of the blots were reacted with the indicated antibodies. (b) Confocal immunofluorescence of MA104 cells transfected with the indicated genes. Tag-VP1 that corresponds to the SV5 tag fused to the VP1 N terminus is shown in green, while NSP5, NSP2 and VP2 are shown in red, as indicated. Single optical sections are shown.
and NSP5 was demonstrated by RNA interference or protein silencing experiments (Campagna et al., 2005; Lopez et al., 2005; Silvestri et al., 2004; Vascotto et al., 2004). The involvement of these two proteins in determining the formation of a basic scaffold for viroplasms was also suggested by co-expression experiments in uninfected cells, which showed that they were able to form VLS, structures morphologically resembling viroplasms (Fabbretti et al., 1999). The capability to form similar structures was also reported as an intrinsic property of the sole NSP5 when a fusion derivative with the EGFP at its N terminus was expressed alone in uninfected cells (Mohan et al., 2003). The results presented here highlight the central role of NSP5 through its ability to form VLS when co-expressed with another viral protein, the core protein VP2. VP2 was found recruited into VLS-VP2i similar to NSP2 in VLS-NSP2i (Fabbretti et al., 1999). Very large inclusions containing both NSP5 and a tagged version of VP2 were previously reported in insect cells (Berois et al., 2003); compared with these, the VLS-VP2i formed in mammalian cells have a more regular shape and, like VLS-NSP2i, resemble the morphology of viroplasms.

An interesting characteristic of the two different VLS inducers, VP2 and NSP2, is the similar effect that both of them have on the phosphorylation status of NSP5. Indeed, NSP5 is significantly hyperphosphorylated in the presence of VP2 showing a pattern of isoforms comparable to that found in infected cells. However, while a correlation between NSP5 hyperphosphorylation and VLS formation would suggest a direct link between the two events, a number of evidence challenge this hypothesis. In fact, four

Fig. 5. NSP5 phosphorylation mutants. (a) Western blot of extracts of cells transfected with the indicated genes and reacted with anti-VP2 or anti-NSP2 (upper parts) and anti-NSP5 (lower parts) sera. (b) Confocal immunofluorescence of wt NSP5 or NSP5 phosphorylation mutants (red) transfected in MA104 cells (vii–ix) or co-transfected with VP2 (i–iii) or NSP2 (iv–vi), as indicated. Single optical sections are shown.
different conditions in which the VP2- or NSP2-mediated hyperphosphorylation was impaired (co-expression with phosphatase PP2A, co-expression with VP1, use of NSP5 phosphorylation mutants or reduced levels of inducers that do not change NSP5 phosphorylation status) still allowed formation of typical VLS. In each condition examined NSP5 hyperphosphorylation induced by VP2 or NSP2 was significantly reduced, and yet VLS formation was essentially not altered. It is therefore reasonable to conclude that the strong NSP5 hyperphosphorylation is not required for its ability to organize into VLS. Yet, since the inhibition of NSP5 phosphorylation was in all cases not complete, it cannot be entirely ruled out that the residual low levels of phosphorylation observed suffice to drive VLS formation. This is in agreement with our previous observations in virus-infected cells, where impaired NSP5 phosphorylation by silencing of protein kinase CK1α did not compromise formation of viroplasms, despite evident alteration of their morphology (Campagna et al., 2007). Despite the evidence of the lack of involvement of NSP5 phosphorylation in the assembly of VLS and viroplasms reported by Campagna et al. (2007) and in the present manuscript, the function of such relevant post-translational modification in the viral replicative cycle remains obscure.

Among the viral proteins found in viroplasms during infection (VP1, VP2, VP3, VP6, NSP2 and NSP5), only VP2 and NSP2 have so far showed the ability to induce NSP5 to arrange into VLS. Indeed, when co-expressed with either VP1 or VP6, NSP5 showed a diffuse distribution. According to these data, in the context of viral infection VP2 and NSP2 almost certainly play an important role in stabilizing the NSP5 arrangement in viroplasms.

In both VLS-VP2i and VLS-NSP2i, the viral polymerase VP1 was found entirely recruited. Targeting of VP1 into VLS might be mediated by the direct interaction with NSP5 (Arnoldi et al., 2007), which, in the context of viral infection, might have the role of recruiting VP1 into viroplasms. The reported interactions of VP1 with NSP2 and VP2 might also contribute to target VP1 to viroplasms (Arnoldi et al., 2007; Kattoura et al., 1994; Zeng et al., 1996, 1998).

Differently from VP1, VP6 was recruited only into VLS-VP2i and not into VLS-NSP2i. This is not surprising because of the well documented interaction of VP6 with VP2 (Charpilienne et al., 2002; Zeng et al., 1996). A similar behaviour has been described for the localization of the bluetongue virus (BTV) protein VP7 (equivalent to RV VP6) into inclusion bodies, which was found to be dependent on the presence of BTV VP3 (equivalent to RV VP2) (Kar et al., 2007). Consistent with our data, co-localization of NSP5, VP2 and VP6 has been described in large inclusions in insect cells infected with recombinant
baculovirus (Berois et al., 2003). The dependence on VP2 for VP6 to be recruited into VLS, further suggests the importance of VP2 for the correct assembly of viroplasms, in agreement with recent reports showing the reduction in the number and size of viroplasms when VP2 was silenced by RNA interference technology (Ayala-Breton et al., 2009; Montero et al., 2008).

The property of VP6 to be recruited only into VLS containing VP2 is of particular interest because it reveals that VLS are not simple aggregates recruiting any available viral protein. This result, along with the evidence that all viroplasmic proteins are recruited into VLS when expressed together, suggest that VLS can be considered as a valid model of viroplasm assembly. A complete validation of VLS as a model needs to address recruitment of viral mRNAs, an exciting challenge for future investigations aimed at assembling true viroplasms. In fact, attempts to target transfected viral mRNAs to viroplasms of infected cells have so far largely failed (Silvestri et al., 2004), with a single exception (Komoto et al., 2006). These observations (Silvestri et al., 2004) suggested the lack of pathways trafficking plus-strand RNAs from the cytosol to viroplasms in virus-infected cells and led to propose the hypothesis of the requirement of the incoming infective particles as nucleation points for the initiation of viroplasm assembly (Silvestri et al., 2004). Alternatively, only the assembly of the very early viroplasms may require recruitment from cytosol of viral mRNAs, which would be later produced in situ by newly formed viroplasmic
Fig. 8. Recruitment of virolomic proteins into VLS. (a) Confocal immunofluorescence of NSP5–EGFP/MA104 transfected with all virolomic protein genes. NSP5 is shown in green (from NSP5–EGFP) and all others in red, as indicated. (b) Confocal immunofluorescence of MA104 cells transfected with all virolomic protein genes, except NSP5. NSP2 is shown in red. In both cases tag-VP1 corresponds to the SV5 tag fused to the VP1 N terminus. Single optical sections are shown.
Recruitment of viroplasmic proteins

DLPs (secondary transcription). In this context, VLS represent an alternative to viroplasms, since they are formed from an initial situation in which there are no incoming infective particles. Thus, VLS may still be suitable for the incorporation of cytosolic viral mRNAs on the way to the de novo construction of true viroplasms and eventually to the formation of infective particles from exogenous mRNAs. Of note, the VLS described here derive from translation of mRNAs that only contain the protein coding sequences of the corresponding viral mRNAs and lack both the 5' and 3' untranslated regions, which are certainly needed for replication and proposed to be also important for packaging based on experimental evidence that the two events are coupled (Patton et al., 2007). Based on this, in order to have functional viroplasms starting from VLS, additional transfection of whole viral mRNAs is needed. In this regard, the successful isolation of recombinant viruses from the family Reoviridae using a system entirely based on in vivo transcribed plasmids, as in the case of orthoreoviruses (Kobayashi et al., 2007), and a system based on transfection of a complete set of in vitro transcribed viral mRNAs, as in the case of BTV (Boyce et al., 2008; Boyce & Roy, 2007), is encouraging.

The results presented highlight the central role of NSP5 in orchestrating redistribution of the viroplasm resident viral proteins. Indeed, NSP5 is the sole component required to organize VLS, likely constituting a scaffold as it does in viroplasms. Interestingly, NSP2, which was also shown to be essential for the assembly of viroplasms (Silvestri et al., 2004), was found to be dispensable for the formation of VLS (such as VLS-VP2i) that were nevertheless able to recruit other proteins (such as VP1 and VP6). This strengthens the hypothesis of NSP5 being the key player for the organization of viroplasms, as reflected by the experiments with all viroplasmic proteins co-expressed in the absence of NSP5 (Fig. 8b).

The participation of cellular proteins in viroplasm assembly has so far not been well documented. Even for other members of the family Reoviridae, the cellular systems assisting formation of cytoplasmic replicative structures are not well characterized, with the sole exception of the microtubule network in the case of viral factories of mammalian orthoreoviruses (Parker et al., 2002). On the contrary, the involvement of the microtubule network has been ruled out for viral inclusion bodies of BTV (Kat et al., 2007) and phytoreoviruses (Wei et al., 2009). Our unpublished observations and recent data (Miller et al., 2010) point to the same conclusion for RV viroplasms, despite reports proposing the need of functional microtubules (Cabral-Romero & Padilla-Noriega, 2006) or association of free tubulin to viroplasms (Martin et al., 2009).

In conclusion, defining the roles of viral proteins, the source and supply mechanisms of viral RNAs to viroplasms and the involvement of cellular components is essential to delineate a comprehensive picture of the complex process of viroplasm assembly during rotaviral infection.

METHODS

Cells, viruses and transient transfections. MA104 cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Invitrogen), 2 mM l-glutamine and 50 μg gentamicin (Invitrogen) ml⁻¹⁻¹.

NSP5–EGFP/MA104 cell line was obtained as previously described (Afrikanova et al., 1996; Eichwald et al., 2004b) and cultured in DMEM complete medium supplemented with 800 μg geneticin (Invitrogen) ml⁻¹⁻¹.

The simian SA11 (G3, P6[1]) strain of RV was propagated in MA104 cells as previously described (Estes et al., 1979; Graham et al., 1987).

For transfection experiments, confluent monolayers of MA104 cells in six-well plates (Falcon) were infected with T7-recombinant vaccinia virus [strain vTF7.3 (Fuerst et al., 1986)] to increase the expression level of proteins encoded by the transfected plasmids. One hour later, cells were transfected with a maximum total of 4 μg per well of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The relative amounts of plasmids were opportunely adjusted as indicated in the figure legend. Transfected cells were harvested at 18 h post-transfection.

Cellular extracts (corresponding to about 5 × 10⁵ cells) were prepared in 100 μl TNN lysis buffer (100 mM Tris/HCl pH 8.0, 250 mM NaCl and 0.5% NP-40) at 4 °C and were subsequently centrifuged at 2000 g for 5 min at 4 °C. Usually, 10 μl of supernatants were used in SDS-PAGE and Western immunoblot analyses.

Construction of plasmids. pT7-vNSP5, pT7-vNSP2, pEGFP–NSP5 (encoding NSP5–EGFP), pcDNA3-SV5-VP1 (encoding tag-VP1) and pcDNA3-VP2 were obtained as previously described (Arnoldi et al., 2002; Eichwald et al., 2002; Fabbretti et al., 1999). The NSP5 gene was derived from the OSU RV strain, the NSP2, VP1 and VP2 genes from the SA11 strain. For the construction of pcDNA3-VP3 and pcDNA3-VP6, the VP3 and VP6 genes were cloned from extracts of SA11 RV-infected cells. The cDNA was obtained by RT-PCR using specific primers for amplifying the ORF of VP3 or VP6 and cloned into pcDNA3 (Invitrogen) (as KpnI–Xhol fragment for VP3 and as KpnI–EcoRV fragment for VP6). The constructs pT7-vNSP5/S67A and pT7-vNSP5a (encoding the phosphorylation mutants of NSP5) and pcDNA3-HA-PP2A/Ca (encoding the 35 kDa catalytic subunit of PP2A) were obtained as previously described (Eichwald et al., 2004a; Pim et al., 2005).

λ-Phosphatase treatment of cellular extracts. Cellular extracts obtained from transfection (20 μl) were incubated with or without 4 μl of λ-phosphatase (400 U μl⁻¹; BioLabs) in buffer for λ-phosphatase treatment (50 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.1 mM EGTA, 2 mM DTT, 0.01% Brij 35) (BioLabs) supplemented with 2 mM MnCl₂. The reaction was incubated for 30 min at 30 °C and was stopped with SDS-PAGE loading buffer (40 % glycerol, 6 % SDS, 125 mM Tris/HCl pH 6.8, 0.04 % bromophenol blue, 5 % β-mercaptoethanol).

SDS-PAGE and Western immunoblot analysis. Cellular extracts were resolved by 10–12 % SDS-PAGE and after electrophoresis transferred to PVDF membranes (Millipore). The membranes were incubated with the following antibodies: anti-NSP2 mouse serum (1:3000), anti-NSP5 guinea pig serum (1:10 000), anti-VP2 mouse serum (1:5000), anti-SV5 (anti-tag) mouse monoclonal antibody (1:10 000), anti-HA rat monoclonal antibody (1:1000; Roche).
and peroxidase-conjugated goat anti-guinea pig (1:10,000; Jackson ImmunoResearch), goat anti-mouse (1:5,000; Jackson ImmunoResearch), rabbit anti-rat (1:2,000; Dako) secondary antibodies. Signals were detected by using the enhanced chemiluminescence system (Pierce).

Sera were produced by immunization of guinea pigs and mice as previously described (Afrikanova et al., 1998; Arnoldi et al., 2007; Gonzalez & Burrone, 1991).

**Immunofluorescence microscopy.** Immunofluorescence experiments were performed as previously described (Eichwald et al., 2002) with the following antibody dilutions: anti-NSP2 guinea pig serum 1:200, anti-NSP5 guinea pig serum 1:1000 or anti-NSP5 mouse serum 1:200, anti-VP6 mouse serum 1:500 or anti-VP2 guinea pig serum 1:200, anti-SV5 (anti-tag) mouse monoclonal antibody 1:1000, anti-HA rat monoclonal antibody (1:100; Roche), anti-VP6 mouse monoclonal antibodies (RV138, 1:50; 4B2D2, 1:1000); rhodamine isothiocyanate-conjugated goat anti-guinea pig (1:200; Jackson ImmunoResearch), fluorescein- or rhodamine isothiocyanate-conjugated goat anti-mouse (1:200; Jackson ImmunoResearch), fluorescein isothiocyanate-conjugated goat anti-rat (1:200; KPL), Alexa-Fluor 647-conjugated goat anti-guinea pig (1:1000; Invitrogen; fluorescence shown in blue in the figures) secondary antibodies. Samples were analysed by confocal microscopy (Zeiss LSM510 equipped with a ×100 NA 1.3 objective or Zeiss LSM510 Meta equipped with a ×63 NA 1.4 objective). Z-stack of 13 images was performed with a z step size of 0.51 µm and was followed by a rendered 3D reconstruction using ImageJ (W.S. Rasband, ImageJ, US National Institutes of Health, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/ plug-in Volume) of Michael Abramoff (Abramoff & Viergever, 2002).

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