Two non-structural rotavirus proteins, NSP2 and NSP5, form viroplasm-like structures in vivo

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In rotavirus-infected cells, the non-structural proteins NSP5 and NSP2 localize in complexes called viroplasms, where replication and assembly occur. Recently, we have demonstrated direct interaction of NSP5 with NSP2, and as a consequence of that, up-regulation of NSP5 hyperphosphorylation. To investigate a possible structural role for the NSP2–NSP5 interaction, we analysed the cytoplasmic distribution of the two proteins in transfected cells by immunofluorescence using specific antibodies. Here we report that NSP2 and NSP5 can drive the formation of viroplasm-like structures (VLS) in the absence of other rotaviral proteins and rotavirus replication. Several NSP5 deletion mutants were constructed and expressed in combination with NSP2. Both the N- and C-terminal domains of NSP5 were found to be essential for VLS formation. Only one mutant, with an internal deletion of residues 81–130, was able to interact with NSP2 to form VLS. Analysis of the phosphorylation capacity of the different mutants in vivo indicated that hyperphosphorylation of NSP5 is necessary, but not sufficient, for VLS formation. Our results suggest a role for the non-structural protein NSP5 in the structure of viroplasms mediated by its interaction with NSP2.

Introduction

Rotaviruses are viruses of the family Reoviridae that contain a dsRNA segmented genome enclosed in an inner core composed of the structural proteins VP1, VP2 and VP3 which is in turn surrounded by a layer of VP6. The outer shell is composed of two other structural viral proteins, VP4 and VP7 (Estes, 1996). Following infection, loss of the outer shell activates the viral transcriptase with the production of mRNAs for all viral genes (Cohen, 1977). Replication and assembly of new particles takes place in the cytoplasm of infected cells, where discrete structures called viroplasms can be found at early times post-infection (Petrie et al., 1982). It is proposed that viroplasms sustain genome replication and assembly. By electron microscopy, single-shelled particles and viral cores have been shown to form in association with viroplasms (Petrie et al., 1984). Several proteins appear to accumulate in these structures during the virus replicative cycle. In particular, by immunofluorescence with specific antibodies, two non-structural proteins, NSP2 and NSP5, were found localized in the viroplasms (Petrie et al., 1984; Welch et al., 1989). NSP2 has ss- and dsRNA binding capacity and can be cross-linked to RNA in vivo by UV treatment of living infected cells (Kattoura et al., 1992; Patton et al., 1993). Furthermore, NSP2 interacts with VP1 and is a component of the replicase complex (Aponte et al., 1996). NSP5, on the other hand, is a glycosylated phosphoprotein encoded in genome segment 11 with a primary sequence characterized by having a high content of serine + threonine (Welch et al., 1989; González & Burrone, 1991). Probably for this reason, NSP5 is strongly phosphorylated in a process that in part occurs through auto-phosphorylation and produces a variety of isoforms differing in their levels of phosphorylation with molecular masses from 26 to 32–34 kDa (Afrikanova et al., 1996; Blackhall et al., 1997; Poncet et al., 1997). We have recently shown, by in vivo cross-linking of infected cells, interaction of NSP5 with NSP2 and the viral polymerase, VP1. Furthermore, we demonstrated in NSP2/NSP5 co-transfection experiments that NSP5 hyperphosphorylation is up-regulated by NSP2 (Afrikanova et al., 1998).

Several reports have described self-assembly of single- and double-layered virus-like particles or replication intermediate particles formed from various combinations of recombinant rotavirus proteins (González & Affranchino, 1995; Crawford et al., 1994; Zeng et al., 1996). We now show that, while expression of NSP2 or NSP5 alone results in a diffuse cytoplasmic distribution, co-expression of both proteins has a
dramatic effect on the localization of either, leading to the formation of viroplasm-like structures (VLS).

**Methods**

- **Cells, viruses and transient transfections.** MA104 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (Gibco). The simian rotavirus strain SA11 was propagated and grown in MA104 cells as described by Estes et al. (1979). Transfection experiments were performed essentially as described by Afrikanova et al. (1998). Briefly, cells were grown on a 35 mm diameter Petri dish, infected with T7–recombinant vaccinia virus (strain vTF7-3) (Fuerst et al., 1986) and 1 h later transfected with 5 µl of Transfectam reagent (Promega) containing a total amount of 2 µg of plasmid DNA (Qiagen purified), as recommended by the supplier. Transfected cells were analysed either by Western immunoblot or immunofluorescence at 16 h post-transfection.

- **Immunofluorescence microscopy and antibodies.** For indirect immunofluorescence microscopy cells were washed twice with PBS–Ca²⁺/Mg²⁺, fixed and permeabilized with ice-cold acetone for 2 min at −20 °C. The coverslips were let to dry, rehydrated in PBS and blocked with 0.25% gelatin (Sigma) in PBS for 30 min at room temperature. Slides were incubated with a guinea-pig anti-NSP5 antisemur (Gonzales & Buronne, 1991) or a mouse anti-NSP2 antisemur (Afrikanova et al., 1998) diluted 1:50 in PBS–3% BSA for 1 h in a moist chamber at room temperature. After three 5 min washings in PBS, the slides were stained for 40 min with FITC-conjugated goat anti-guinea-pig (Sigma) or RITC-conjugated goat anti-guinea-pig (Sigma) and FITC-conjugated goat anti-mouse (Dako) antibodies, washed and mounted with KPL mounting medium. Samples were analysed by either conventional immunofluorescence (Nikon) or confocal microscopy (Molecular Dynamics and Bio-Rad Microrandance).

- **Plasmid constructs.** pT7v-NSP2, pT7v-NSP5, pT7v-AN18, pT7v-ΔN33, pT7v-AN80, pT7v-AC29, pT7v-AC48, pT7v-AC68, pT7v-Δ34–80, pT7v-Δ81–130 and pT7v-Δ131–179 have been either reported or obtained as in Afrikanova et al. (1998). Both NSP5 and NSP2 sequences were derived from the SA11 rotavirus strain. Internal deletion mutants were obtained by PCR using specific internal primers (5' TGTCTTACAGATITTCCAGAAAAG 3' and 5' GAATAATCTTTAAGAACAAATGTGCGAG 3'). 5' TCACATGATCTAATCGAAAA 3' and 5' ATTAGATGATTCGAGATATATAAAAAAAGCGAATTAC 3', 5' TGGCGATAACGAGTGGTACG 3' and 5' ATCTCAACTTAGACAAG 3', to amplify Δ34–80, Δ81–130 and Δ131–179, respectively. N-terminal deletion mutants were amplified with the unique 3' primer 5' GATACATTTACAAATCTTGATC 3' and 5' primers 5' GTTACCATGATTGATGGAG3' and 5' GGTACCAGATGGTTAAGACAAATG3' for AN33 and AN80 mutants, respectively. C-terminal deletion mutants were amplified with the unique 5' primer 5' GATACATTTACAAATCTTGATC 3' and 3' primers 5' GATACATTTACAAATCTTGATC 3' and 5' GGATACATTTACAAATCTTGATC 3' and 5' GGATACATTTACAAATCTTGATC 3' to amplify ΔC18, ΔC29, ΔC48 and ΔC67 fragments, respectively. All constructs were cloned as KpnI–BamHI restriction fragments in pcDNA3 (Invitrogen) and checked by complete sequencing of both strands.

- **32P labelling, immunoprecipitations and Western immunoblot analysis.** 32P labelling of transfected cells was performed at 13 h post-transfection and cell lysates were immunoprecipitated and analysed in SDS–12% polyacrylamide gels (Laemmli et al., 1970) essentially as described by Afrikanova et al. (1998). For Western immunoblot analysis, lysates were prepared at 16 h post-transfection, resolved on an SDS–12% polyacrylamide gel and transferred onto a PVDF membrane (Millipore). An anti-NSP5 guinea-pig serum was used at a dilution of 1:1000; anti-guinea-pig HRP-conjugated antibody (Dako) was used as a secondary antibody, and developed by the ECL system (Amersham).

**Results**

**In vivo formation of viroplasm-like structures (VLS)**

In virus-infected cells NSP2 and NSP5 are localized in cyttoplasmic viroplasms (Fig. 1a, b). We have recently demonstrated interaction of NSP5 with NSP2 by co-immunoprecipitation of both proteins from extracts of chemically (DSP)-cross-linked or UV-treated virus-infected cells. Furthermore, we have also shown that NSP5 hyperphosphorylation in vivo is up-regulated by NSP2 (Afrikanova et al., 1998).

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**Fig. 1.** Localization of rotavirus NSP2 and NSP5. The two proteins were detected by immunofluorescence microscopy using antibodies specific for NSP2 or NSP5 as indicated, in cells infected with SA11 rotavirus (a, b), transfected with either NSP2 (c, d) or NSP5 (e, f), or co-transfected with NSP2 and NSP5 (g, h).
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Because of this interaction and the fact that the two proteins co-localize in viroplasms of virus-infected cells, we decided to investigate their intracellular localization when both proteins were co-expressed in the absence of any other rotavirus protein and rotavirus replication. To achieve this, we used the T7–vaccinia virus expression system to co-transfect into MA104 cells plasmids containing the complete coding region of NSP5 and NSP2 under the control of the T7 polymerase promoter.

As shown in Fig. 1, immunofluorescence detection of NSP2 and NSP5 with specific antibodies shows a diffuse cytoplasmic distribution when either protein is expressed alone (Fig. 1c–f). However, when both proteins are co-expressed, formation of VLS becomes evident (Fig. 1g, h). These VLS can be visualized with both anti-NSP2 and anti-NSP5 antibodies similarly to what is observed in virus-infected cells (Fig. 1a, b).

Confocal microscopy with double immunofluorescence for NSP2 and NSP5 revealed the co-localization of both proteins in viroplasms as well as in VLS, thus confirming that the VLS observed are the consequence of NSP2–NSP5 interaction (Fig. 2a). In addition, serial confocal images suggest a near spherical shape of VLS (Fig. 2b).
Even though VLS obtained by co-expression of NSP5 and NSP2 are similar to viroplasms of rotavirus-infected cells, VLS appear as ‘empty’ structures with NSP2 and NSP5 co-localizing on the surface, while viroplasms show punctuated staining for both NSP2 and NSP5. The fact that VLS appear as empty structures may reflect the absence of viral genomic RNA and structural core proteins in the transfected cells.

**N- and C-terminal domains of NSP5 are required for VLS formation**

In order to map the NSP5 domains relevant for the interaction with NSP2 and VLS formation, we constructed a number of NSP5 deletion mutants and tested them for their capacity to form VLS when co-expressed with NSP2. A schematic representation of the different NSP5 mutants is shown in Fig. 3.

Mutants lacking either N-terminal (∆N33, ∆N80) or C-terminal (∆C18, ∆C29, ∆C48 and ∆C68) regions were completely inactive in forming VLS. Fig. 4 shows one example with mutant ∆N33, in which diffuse localization is observed regardless of whether the mutated NSP5 is expressed alone or together with NSP2 (Fig. 4a, b). Similar results were obtained with mutants ∆N80, ∆C18, ∆C29, ∆C48 and ∆C68 (data not shown). On the other hand, the internal deletion mutant ∆d81–130, which lacks the domain corresponding to aa 81–130, produced VLS, although smaller than the ones obtained with wt NSP5 (Fig. 4c, d), whereas two other internal
Discussion

In rotavirus-infected cells, NSP5 and NSP2 localize in the cytoplasm in multiprotein complexes called viroplasms, or virus factories, where replication of the virus occurs. Here we demonstrate that, in transfected cells, NSP2 and NSP5 can drive the formation of viroplasm-like structures (VLS) in the absence of any other rotaviral protein and of rotavirus replication. This result is particularly interesting if considered in the context of NSP5 post-translational modifications. NSP5 is a phosphoprotein and an O-glycoprotein (Welch et al., 1989; González & Burrone, 1991). This latter modification comprises the addition of monomeric residues of N-acetylglucosamine (GlcNAc) to serine or threonine residues and is characteristic of many proteins that localize in the cytoplasm or are transported to the nucleus. This type of glycosylation is also involved in regulating the degree of phosphorylation of many serine/threonine phosphoproteins (Chou et al., 1995; Snow & Hart, 1998). Indeed, this appears to be the case for NSP5; we have shown that NSP5 is capable of being phosphorylated at a number of residues, giving rise to a variety of isoforms that have altered migration in SDS–PAGE. The most-phosphorylated isoforms are also the ones that contain least GlcNAc (Afrikanova et al., 1996). We show here that in addition to wt NSP5, the two other deletion mutants that are positive for VLS are the only ones that can also be phosphorylated. We have recently demonstrated that in infected cells NSP5 interacts with NSP2 inducing activation of NSP5 hyperphosphorylation (Afrikanova et al., 1998). The co-localization of the two proteins and the formation of VLS in vivo appear to be the result of the fine cooperation between

Deletion mutants, ∆d34–80 (Fig. 4e, f) and ∆d131–179 (Fig. 4g, h), lacking, respectively, domains 34–80 and the basic-acid basic 131–179, did not form VLS when co-expressed with NSP2. ∆d131–179 did, however, show a non-homogeneous distribution with irregular spots and short spikes even in the absence of NSP2. On the other hand, mutant ∆d34–80, even though sensitive to the presence of NSP2, produced irregular structures rather than the spherical VLS.

NSP5 phosphorylation is necessary but not sufficient for VLS formation

Since NSP5 phosphorylation and VLS formation are both related to the NSP5–NSP2 interaction, we investigated the capacity of the different NSP5 mutants to be phosphorylated in vivo. As we have previously reported, ∆N33 is hyperphosphorylated when expressed on its own, but does not have NSP2 up-regulated phosphorylation, whereas the C-terminal deletion mutant ∆C68 is not phosphorylated, either in the presence or in the absence of NSP2 (Afrikanova et al., 1998). Hyperphosphorylation of wt NSP5 and its deletion mutants (which is λ-Pase sensitive) can be easily assessed because it largely affects their migration on SDS–PAGE (Afrikanova et al., 1996, 1998). However, since NSP5 can also be phosphorylated without changes in mobility (Afrikanova et al., 1996), in order to assess the degree of phosphorylation of the different mutants we performed Western immunoblot analysis (Fig. 5a) and 32P incorporation in vivo labelling and immunoprecipitation (Fig. 5b) of transfected cells.

∆N33 showed a strong hyperphosphorylation as evidenced by the reduced mobility (Fig. 5a, lane 3) and 32P incorporation (Fig. 5b, lane 2). All other N- and C-terminal deletion mutants (∆N80, ∆C18, ∆C29, ∆C48 and ∆C68) which were unable to form VLS did not show a change in mobility, even though ∆N80, ∆C18 and ∆C29 become phosphorylated as seen in Fig. 5 (b), lanes 3, 6 and 7. On the other hand, mutant ∆d81–130, which was active in VLS formation, was also strongly phosphorylated, producing a shift from around 21 kDa to hyperphosphorylated forms of up to 29–30 kDa (Fig. 5a, lane 9). These low mobility bands were also the ones with stronger 32P incorporation (Fig. 5b, lane 4). Interestingly, neither of the two other internal deletion mutants unable to form VLS, ∆d34–80 and ∆d131–179, were phosphorylated (Fig. 5a, lanes 8 and 10, Fig. 5b, lanes 5 and 10). In addition, none of the mutants described showed NSP2 up-regulated phosphorylation (not shown).
NSP5 hyperphosphorylation and NSP5–NSP2 interaction. Analysis of NSP5 deletion mutants expressed in vivo in combination with NSP2, and their capacity to become phosphorylated, strongly suggest that although phosphorylation of NSP5 is necessary for VLS formation (wtNSP5 and mutants incorporated in VLS are phosphorylated), it is not sufficient since mutant ΔN33, which does not form VLS, is hyperphosphorylated. In addition, other mutants which were unable to form VLS such as ΔN80, ΔC18 and ΔC29 were also labelled with $^{32}$P but did not show a mobility change. On the other hand, NSP2 appears to be essential for VLS formation, since neither wtNSP5 nor any of its mutants were able to produce VLS when expressed alone. It has been shown that in cells infected with a ts-mutant for the VP6 gene, at the non-permissive temperature VP6 but not NSP2 fails to localize in viroplasms (Mansell et al., 1994).

The results obtained with mutant ΔN33 suggest that the N-terminal region of NSP5 is a likely candidate domain for the interaction with NSP2. It also appears to play a regulatory role in NSP5 phosphorylation. Indeed, its deletion leads to increased phosphorylation of NSP5, unresponsiveness to NSP2-induced hyperphosphorylation and lack of VLS formation. Furthermore Δd81–130, the only other mutant that is able to form VLS and which contains an intact N33 domain, becomes hyperphosphorylated in vivo generating species of highly decreased mobility in SDS–PAGE. Hyperphosphorylation of Δd81–130, however, is not sufficient to organize it into discrete cytoplasmic structures; this requires expression of NSP2 also. Further, the distribution of mutant Δd34–80 is sensitive to the presence of NSP2 but this mutant generates irregular structures which do not resemble the spherical VLS and it is not phosphorylated. Further experiments are needed.

Fig. 5. Phosphorylation of NSP5 and mutants. (a) Western immunoblotting of each independently transfected construct. Filled arrowheads indicate $^{32}$Pase-sensitive hyperphosphorylated species which have a reduced gel mobility.

(b) Immunoprecipitation of cells transfected with the indicated mutant constructs and labelled in vivo with $^{32}$P.

Relative molecular masses in kDa are shown to the left.
to establish whether, for wtNSP5, there is differential participation of the various isoforms in the formation of VLS.

The confocal microscopy analyses presented suggest that VLS are spheres of different size which are the precise site of co-localization of NSP2 and NSP5. From these studies we can underline some differences between the viroplasms and VLS. While VLS appear as ‘empty’ structures with an inner part lacking NSP2 and NSP5, rotavirus viroplasms, if observed at high magnification, show punctuated staining coincident for NSP2 and NSP5. Viroplasms have been shown to be electron-dense structures when observed by electron microscopy (Petrie et al., 1982, 1984).

We are grateful to Massimo Righi (SISSA, Trieste) and Dino Sharma (Bio-Rad, Bristol, UK) for helpful assistance with the confocal microscopy.

References


Received 19 June 1998; Accepted 26 October 1998