Rotavirus Nonstructural Protein NSP5 Interacts with Major Core Protein VP2

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Rotavirus is a nonenveloped virus with a three-layered capsid. The inner layer, made of VP2, encloses the genomic RNA and two minor proteins, VP1 and VP3, with which it forms the viral core. Core assembly is coupled with RNA viral replication and takes place in definite cellular structures termed viroplasms. Replication and encapsidation mechanisms are still not fully understood, and little information is available about the intermolecular interactions that may exist among the virolas proteins. NSP2 and NSP5 are two nonstructural viroplasmic proteins that have been shown to interact with each other. They have also been found to be associated with precoore replication intermediates that are precursors of the viral core. In this study, we show that NSP5 interacts with VP2 in infected cells. This interaction was demonstrated with recombinant proteins expressed from baculovirus recombinants or in bacterial systems. NSP5-VP2 interaction also affects the stability of VP6 bound to VP2 assemblies. The data presented showed evidence, for the first time, of an interaction between VP2 and a nonstructural rotavirus protein. Published data and the interaction demonstrated here suggest a possible role for NSP5 as an adapter between NSP2 and the replication complex VP2-VP1-VP3 in core assembly and RNA encapsidation, modulating the role of NSP2 as a molecular motor involved in the packaging of viral mRNA.
MATERIALS AND METHODS

Cells and virus. The RF strain of rotavirus was propagated in MA104 cells in Eagle’s minimum essential medium in the presence of tripsin (0.5 μg/ml). Infections were carried out at a multiplicity of infection of 10 PFU/cell. S9 cells infected with each recombinant baculovirus at a multiplicity of infection of 5 PFU/cell were incubated in Hink’s medium supplemented with 1% fetal calf serum. Recombinant baculovirus encoding the following rotavirus proteins were used: VP1 (pVL941/RF-1) (9), VP2 (BacRF2A) (22), VP2 with the first 92 amino acids deleted (Bac29VP2) (32), green fluorescent protein (GFP) fused to VP2 (BacGFP-92VP2) (7), VP3 (BacRF3) (A. Charpliullene et al., unpublished data), VP6 (BacRF6) (22), and NSP5 (BacNSP5RF) (27). NSP5 fused to the carboxyl terminus of glutathione S-transferase (GST) was expressed in E. coli BL21/DE3 transformed with recombinant plasmid pET41a-RF11.

Purification of rotavirus double-layered VLP. Double-layered virus-like particles (VLP) composed of VP1, VP2, VP3 and VP6 (VLP1/2/3/6); and of VP2 and VP6 (VLP2GP/6); of VP6 (VLP2/6); of VP2 with the amino terminus of VP1, VP2, VP3 (VLP1/2/3/6); of VP2 fused to GFP and VP6 (VLP2GFP/6) were produced and purified as described previously (22).

Briefly, S9 cells were collected at 7 days postinfection and treated with Freon 113. Cell extract was then mixed with CsCl, adjusted to obtain a refraction index B 1.360, and centrifuged for 16 h at 35,000 rpm in a Beckman SW55 rotor. The supernatant was incubated with glutathione-agarose beads overnight at 4°C. The beads were washed with phosphate-buffered saline containing 10 mM dithiothreitol, 1% Triton X-100, and 1% Tween 20. The lysate was clarified at 14,000 × g for 15 min, and the supernatant was incubated with glutathione-agarose beads overnight at 4°C. The beads were washed with phosphate-buffered saline, and proteins bound to glutathione-agarose were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining.

Coimmunoprecipitation and immunostaining. Rotavirus-infected MA104 cells (3 × 10⁶) were collected at 5 h postinfection and lysed in 0.5 ml of 50 mM Tris-HCl (pH 8)–125 mM NaCl–30 mM KCl–0.1% Triton X-100–0.05% SDS–2 mM EDTA–2 μg of aprotinin/ml (IP buffer). Cell debris was pelleted by centrifugation at 100,000 × g for 15 min. Monoclonal antibody (MAb) 158G37 directed against NSP5 (27) (1 μl of ascitic fluid) was added to 300 μl of cell lysate supernatant and incubated overnight at 4°C. Alternatively, S9 cell lysates supernatant (150 μl) prepared as described above from cells infected with various recombinant baculoviruses were incubated with MAb 158G37. Complexes were precipitated with protein A bound to Sepharose 4B and washed with IP buffer or HB depending on whether they were obtained from MA104 or S9 cells, respectively.

Immunoprecipitated proteins were immunodetected after SDS-PAGE analysis and blotting on polyvinylidene difluoride membranes by transverse electrophoresis in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11, and viral cores were purified as previously described (4). Glutathione-agarose beads loaded with recombinant GST-NSP5 were incubated overnight with 80 μg of CsCl gradient-purified VLP, DLP, or cores in a volume of 500 μl of 50 mM KCl–5 mM MgCl₂–20 mM HEPES (pH 7)–0.5% Triton X-100 at 4°C. The beads were washed with the same buffer, and protein complexes were analyzed by SDS-PAGE, Coomassie blue stained, and further identified by immunostaining as described above.

Alternatively, 100 μl of NSP5-containing lysates prepared from BacNSP5RF-infected S9 cells as described above were mixed with 80 μg of CsCl-purified rotavirus particles (VLP2/6, VLP2GFP/6, VLP2A92/6, VLP1/2/3/6, and DLP), resuspended in HB, and incubated for 1 h on ice. Samples were layered onto a 4.5-m CsCl density gradient and spun in a Beckman ultracentrifuge for 16 h at 35,000 rpm at 4°C in a SW55 rotor. As control, lysate prepared from S9 cells infected with a recombinant baculovirus allowing the expression of NSP2 was used under the same conditions.

RESULTS

NSP5 and VP2 coimmunoprecipitate. To look for interactions between NSP5 and VP2, lysates of MA104 cells infected with rotavirus for 5 h were incubated with anti-NSP5 MAb. Immunoprecipitated protein complexes were analyzed by Western blotting with MAb directed against either VP2 or VP6. As shown in Fig. 1, VP2 was detected in these complexes (lanes RF). By contrast, VP6 was not detected in the immunoprecipitated complexes containing NSP5. Mock-infected cells were used as controls (lanes M). Reverse experiments, i.e., immunoprecipitation with anti-VP2 MAb and Western blotting with anti-NSP5, gave lower signals (not shown). This result could be explained by the possibility that only a subfraction of NSP5 is complexed with viral cores.

Interaction between NSP5 and VP2 was confirmed by coexpression of both proteins in insect cells (Fig. 2). Using the same assay as described above, we confirmed the presence of VP2 in the complexes immunoprecipitated with a MAb directed against NSP5 (Fig. 2, lane 2). The same results were obtained when the deletion mutant 92VP2 was substituted for wild-type VP2, indicating that the interaction between the two proteins is not mediated by the amino end of VP2 (not shown).

Rotavirus proteins are all highly expressed in the baculovirus system, and it is possible to express more than two proteins in insect cells. We used this versatile expression system to study the influence of other rotaviral virophilic proteins on VP2-NSP5 interactions. As shown in Fig. 2, interaction between VP2 and NSP5 is not prevented when NSP2 (lane 3), VP6 (lane 5), or both (lane 6) are coexpressed with VP2 and NSP5.
in insect cells. In the absence of infection with the NSP5-expressing baculovirus, VP2 was not detected (lanes 1 and 4).

**NSP5 modifies the subcellular distribution of VP2.** In rotavirus-infected cells, NSP5 and VP2 are colocalized in viroplasms (14). To explore the possible role of the interaction shown above in the colocalization of VP2 and NSP5 in the viroplasms, we used insect cells infected with recombinant baculoviruses. We used amino-terminally deleted VP2 fused to GFP (GFP-H900492VP2) instead of VP2, since this fusion protein has the same assembly properties with itself and with VP6 as wild-type VP2 (7). As shown in Fig. 3A, expression of GFP-\(\Delta 92\)VP2 in Sf9 cells resulted in evenly distributed small fluorescent inclusions. In contrast, coexpression of GFP-\(\Delta 92\)VP2 and NSP5 resulted in fewer but much larger fluorescent VP2 inclusions (Fig. 3B). These large inclusions contained both VP2 and NSP5 (Fig. 3C1 to C3). Confocal microscopy examination of these insect cells demonstrated that small inclusions present when GFP-H900492VP2 was expressed alone clustered together only when NSP5 was coexpressed with GFP-\(\Delta 92\)VP2. The pattern of fluorescence of GFP-\(\Delta 92\)VP2 was not modified when it was coexpressed with NSP2 (Fig. 3D). Interestingly, the distribution of GFP-\(\Delta 92\)VP2 was also modified when VP6, which is known to interact with VP2, was coexpressed (Fig. 3E). The large inclusions characteristic of coexpression of GFP-\(\Delta 92\)VP2 and NSP5 were not modified when the three proteins VP6, NSP5, and GFP-\(\Delta 92\)VP2, were coexpressed (Fig. 3F).

EM of thin sections of Sf9 cells infected with baculovirus expressing VP2 clearly showed inclusions containing spherical structures evoking VP2 pseudocores (Fig. 4A). In contrast, when VP2 and NSP5 were coexpressed, inclusions were larger and contained less-structured viral assemblies (Fig. 4B).

**Recombinant NSP5 binds cores and VLP but not DLP.** The experiments described above do not provide information about the polymeric structure of VP2 (monomeric or polymeric) that interacts with NSP5. To clarify this point, purified recombinant GST-NSP5 bound to glutathione-agarose beads was incubated with similar amounts of various types of purified viral particles, namely DLP, cores, VLP2/6, and VLP1/2/3/6. Cores and DLP were derived from purified virus and contain genomic RNA. Recombinant VLP2/6 and VLP1/2/3/6 were purified from insect cells. The input of the various types of particles used in the pull-down assay is shown in Fig. 5 (top panel). Proteins bound to GST-NSP5 glutathione-agarose beads were analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 5, middle panel). Figure 5 (middle panel) clearly illustrates that DLP were not pulled down by NSP5 bound to agarose beads. In contrast, NSP5 beads pulled down cores, VLP2/6, and VLP1/2/3/6. It can be observed that the ratio of VP2 bound to VP2 present in the particle input is always lower when VP6 is present in the particles interacting with NSP5 beads (VLP2/6 and VLP1/2/3/6). When VP6 is absent, i.e., in cores, the intensity of the VP2 band is maximum, suggesting that the binding of particles to NSP5 is optimal. This assay also showed that the binding of VLP2/6 and VLP1/2/3/6 to NSP5 resulted in a

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change of the stoichiometry of VP6 in the pulled down material (compare the intensity of VP6 bands with that of VP2 bands in the upper and middle panels of Fig. 5). Immunostaining with a MAb directed against VP2 confirmed the identity of the protein migrating as a 90-kDa band and the absence of VP2 when DLP were incubated with NSP5 beads (Fig. 5, lower panel). As a control, pull-down experiments were performed with GST-bound beads. None of the various types of viral particles showed any binding to the GST beads (data not shown).

The pulling down of VLP2/6 and the absence of pulling down of DLP by GST-NSP5 beads could be due either to the absence of a limited number of VP6 trimers on VLP or to a stronger interaction between VP2 and VP6 in DLP than in VLP. Since both situations provide access to VP2, we compared the stabilities of the two types of viral particles at high pH in order to clarify this point. As shown in Fig. 6A, DLP were essentially resistant to such a treatment, but VLP2/6 were transformed into pseudocores (Fig. 6B).

**NSP5 dislodges VP6 from purified VLP2/6.** To characterize the complexes made of VP2 and NSP5, we analyzed purified VLP2/6 incubated with crude NSP5 from recombinant baculovirus-infected insect cell lysate by centrifugation in a CsCl gradient. A control experiment was performed with an Sf9 cell lysate containing NSP2. As shown in Fig. 7, incubation of purified VLP2GFP/6 with BacNSP2-infected Sf9 cell lysate did not change the density of VLP (Fig. 7, left panel, band a). In contrast, when the Sf9 cell lysate contained NSP5, an additional band was observed. The upper band (Fig. 7, left panel, band b) is similar to the band (band a) observed in the control, but the lower band contained flocculate fluorescent material (Fig. 7, left panel, band c). Both bands were fluorescent and thus contained GFP-Δ92VP2. Proteins present in these bands were analyzed by SDS-PAGE. As shown in Fig. 7 (middle...
Viral factories referred to as viroplasms are observed in the cytoplasm of rotavirus-infected cells as early as 5 h postinfection. They contain several structural and nonstructural viral proteins, including NSP5 and VP2. In this study we have shown a direct interaction between VP2 and NSP5 early after infection. This interaction was confirmed in insect cells when VP2 and NSP5 were coexpressed in the absence of another viral protein and viral RNA. In this expression system, VP6 and NSP2, which are both components of viroplasm, did not inhibit VP2-NSP5 interactions. We did not observe interactions between NSP2 and VP2 in insect cells. NSP5 interaction with VP2 is not required for assembly of inner capsid proteins (VP1 and VP3 [possibly viroplasmic]) or outer capsid proteins (VP7 and VP4 [not viroplasmic]), since it has been demonstrated that all of these proteins assemble in the absence of NSP5 in insect cells (10, 32). We also demonstrated that NSP5 interacts with purified polymeric assemblies of VP2, including cores, VLP2/6, and VLP1/2/3/6.

In rotavirus-infected MA104 cells, most of the NSP5 is hyperphosphorylated, and its interaction with VP2 as demonstrated in these cells is probably due to this form. In this study, we also used nonphosphorylated NSP5 derived from E. coli and phosphorylated (but not hyperphosphorylated) NSP5 derived from insect cells. It was possible to demonstrate the interaction of VP2 with all forms of NSP5. This indicates that the phosphorylation state of NSP5 is not essential for the interaction, but it cannot be excluded that the interaction could be modulated by various degrees of phosphorylation of NSP5. The amino-terminal domain of VP2 is not necessary for the interaction, since a deletion mutant lacking the first 92 amino acids of VP2 interacted with NSP5 as well as wild-type VP2 did. The amino end of VP2 corresponds to the nucleic acid binding domain of VP2. This also suggests that the interaction between the two proteins does not involve nucleic acid.

It has previously been shown that NSP5 expressed alone is...
evenly distributed into the cytoplasm (1, 27). In this study we demonstrated that VP2 alone is not evenly distributed in insect cells and that coexpression of both proteins resulted in a dramatic change in the size of VP2 inclusions. The increased size of inclusions could simply result from the accumulation of NSP5 and VP2 proteins in the same area. NSP5 is a small oligomeric protein (27) that could link VP2 inclusions in large aggregates. The increased size of VP2 inclusions when NSP5 is present could also be the consequence of a conformational change of VP2 induced by NSP5. This hypothesis is supported by EM observations of an altered morphology of VP2 pseudocores when NSP5 is coexpressed with VP2 in insect cells. A similar increase in the size of viral inclusions has been observed when the core protein of hepatitis C virus is coexpressed with NSP5A in COS-7 cells (13).

In this study, we have shown that NSP5 interacts with VP2 and that VP6 hinders this interaction. The modulation of VP2-NSP5 interaction by VP6 could be due to a strong interaction between VP6 and VP2 that itself could be dependent on the presence of RNA inside viral particles. The interaction between VP2 and VP6 is stronger in DLP than in VLP2/6 as demonstrated by the respective resistances of the two types of particles to high-pH treatment. It can be hypothesized that the binding of NSP5 to core RI that do not yet have their complete set of genomic RNA segments prevents the binding of VP6 that would result in a defective particle containing a partial set of genomic segments, based on the observation that purified DLP did not interact with NSP5. The possibility that some VP6 trimers are absent and allow access to VP2 in VLP2/6 cannot be totally excluded either. The binding of NSP5 to rotavirus cores is reminiscent of the binding of μNS to reovirus cores (6). In reovirus-infected cells, μNS reduces the formation of recoated cores that are transcriptionally active, by the binding of outer capsid proteins μ1 and σigma 3 (which could be considered VP6 and VP7 counterparts, respectively), whereas NSP5, which dislodges VP6 from VLP and binds to transcriptionally inactive cores, could prevent an early start of transcriptase activity of newly formed DLP. It is therefore possible that μNS and NSP5 could both play a role in the delicate switch between transcriptase and replicase activities.

It has already been demonstrated that NSP5 interacts with NSP2 (1, 27). The data presented here are the first evidence of an interaction between NSP5 and a structural protein and the first evidence of an interaction between VP2 and a nonstructural protein. This interaction could be significant in the context of RNA replication and capsid morphogenesis. In a recently proposed model (17, 24), interaction between NSP5 and NSP2 in a complex consisting of VP1, VP3, VP2, NSP2, NSP5, and RNA was considered. Direct interaction between VP2 and NSP5, as demonstrated here, would modify this model and place NSP5 as an intermediate between VP2 and NSP2. Moreover, our results suggested that the competition between VP6 and NSP5 for VP2 could release the NSP2-NSP5 complex from the core RI (26). This could most likely occur at the edge of the viroplasm, where VP6 seems to accumulate (14).

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