RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle

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Rotavirus genomes contain 11 double-stranded (ds) RNA segments. Genome segment 11 encodes the non-structural protein NSP5 and, in some strains, also NSP6. NSP5 is produced soon after viral infection and localizes in cytoplasmic viroplasm, where virus replication takes place. RNA interference by small interfering (si) RNAs targeted to genome segment 11 mRNA of two different strains blocked production of NSP5 in a strain-specific manner, with a strong effect on the overall replicative cycle: inhibition of viroplasm formation, decreased production of other structural and non-structural proteins, synthesis of viral genomic dsRNA and production of infectious particles. These effects were shown not to be due to inhibition of NSP6. The results obtained strengthen the importance of secondary transcription/translation in rotavirus replication and demonstrate that NSP5 is essential for the assembly of viroplasms and virus replication.

INTRODUCTION

Rotaviruses have a genome composed of 11 segments of double-stranded RNA (dsRNA). Virus replication is entirely cytoplasmic and takes place within viroplasm, discrete structures formed at early times post-infection. The non-structural protein NSP5, encoded by genomic segment 11, is found in viroplasm of virus-infected cells. The role of NSP5 remains to be elucidated, although a number of biochemical characteristics have been described, including its O-glycosylation, hyperphosphorylation and interaction with NSP2 and with the inner core protein VP2 (Welch et al., 1989; Chen et al., 1990; Gonzalez & Burrone, 1991; Kattoura et al., 1992, 1994; Afrikanova et al., 1996, 1998; Aponte et al., 1996; Poncet et al., 1997; Taraporewala et al., 1999; Taraporewala & Patton, 2001; Eichwald et al., 2002; Berois et al., 2003). We have recently reported that NSP5 is also essential for viroplasm assembly (Vascotto et al., 2004). In some group A rotavirus strains, such as SA11, the genome segment 11 (gs11) also encodes a second non-structural protein, termed NSP6, of 91 residues, from a different ORF (Matton et al., 1991). In the porcine OSU strain, however, this second ORF encodes a truncated protein of 51 amino acids (ORF2) (Gonzalez & Burrone, 1989).

Due in part to the lack of a reverse genetics system in rotavirus, no clear function in the viral replicative cycle has yet been found for NSP5. Here, we used the RNA interference strategy directed towards gs11 mRNA to demonstrate that NSP5 is an essential protein for the formation of viroplasm and for virus replication.

METHODS

Cell culture and viruses. C7 and NSP2-EGFP stable cell lines were obtained and cultured as described previously (Afrikanova et al., 1996; Eichwald et al., 2004). SA11 and OSU rotaviruses were propagated in MA104 cells as described by Estes et al. (1979). Virus titres were determined by immunofluorescence on MA104 cells with anti-NSP2 antibody or by direct determination of viroplasm in the NSP2-EGFP stable cell line.

Small interfering (si) RNAs and transfection. The siRNAs were chemically synthesized with a 3′ TT deoxynucleotide overhang on both strands and obtained as annealed duplexes (IBA). A fluoresceinated siRNA against SA11 segment 11 (siSA11-fluo) with 6-FAM on the 5′ end of the sense strand was obtained as an annealed duplex (Qiagen). Approximately 1·5 × 10⁵ cells were transfected with 2 μg siRNAs in 1 ml serum-free medium containing 5 μl Transfectam reagent (Promega). After 6 h at 37 °C, cells were washed twice with serum-free medium and incubated for an additional 66–72 h in medium supplemented with 10% fetal bovine serum (Gibco). This time point was found to be the optimum for the interfering effect (Dector et al., 2002). All experiments with siRNA were repeated several times and the data shown are representative of the results obtained.

Western immunoblot analysis. Cells were lysed and Western blots performed as described previously (Eichwald et al., 2002). SDS-PAGE (Laemmli, 1970) was done with equal amounts of proteins. Membranes were incubated with guinea pig sera against viral proteins NSP5, NSP2, VP7 and VP1, followed by goat anti-guinea pig HRP-conjugated antibody (KPL), and developed with the ECL.
system (Amersham). Antibodies for NSP5 and NSP2 were prepared as described by Eichwald et al. (2002). Anti-VP7 and anti-VP1 sera were obtained by immunization of guinea pigs with glutathione S-transferase- and His-tagged fusion proteins, respectively, essentially as described by Gonzalez & Burrone (1991).

**Immunofluorescence.** Immunofluorescence was performed essentially as described previously (Eichwald et al., 2002). Slides were mounted with ProLong mounting medium (Molecular Probes) and acquired with cool SNAPs system using a fluorescence microscope (DMLB; Leica). Around 500 cells were counted per experimental point and the experiment was repeated at least three times. Viroplasm-positive cells were considered those with two or more dots per cell.

**Viral genomic dsRNA.** Equal numbers of rotavirus-infected cells were collected and total genomic dsRNA was prepared as described previously (Chen et al., 1990). Samples were electrophoresed in a 10% polyacrylamide gel, 0.75 mm thick, for 15 h at 20 mA and bands were visualized with ethidium bromide.

**RESULTS**

We designed two double-stranded 19 bp siRNAs to target rotavirus gs11 mRNA, between nucleotides 85 and 103 within the NSP5 and NSP6 (or ORF2) coding region, for two different rotavirus strains (simian SA11 and porcine OSU), which differ in that region in a single nucleotide in position 93 (Fig. 1a). The two synthetic siRNAs (termed si/SA11 and si/OSU) contain in each strand a 3′ end with a TT deoxyribonucleotide overhang (Fig. 1b). To test the activity of the siRNAs, we transfected them into MA104 cells, which were infected 72 h later with both rotavirus strains. When NSP5 expression was analysed at 5 h post-infection, a large reduction in the amount of SA11 or OSU NSP5 was observed in cells transfected with the corresponding siRNA, while practically no effect was observed in cells infected with either virus that had received the non-homologous siRNA (Fig. 1c). At longer times post-infection (15 h), a significantly larger amount of NSP5 was observed, probably due to cells that did not uptake the siRNA or to the fact that continuous production of viral mRNA overrides the interfering activity (Figure 1d). These results indicated that both siRNAs were highly efficient in specifically targeting the homologous gs11 mRNA, producing NSP5 protein-knockout phenotypes. We also carried out Western-blot analysis for other viral proteins that do (VP1, NSP2) and do not (VP7) localize in viroplasms. A strong inhibition of the accumulation of all three viral proteins took place.
concomitantly with the reduction of NSP5, while this was not the case for the control cellular protein actin (Fig. 1c).

Since NSP5 localizes in cytoplasmic viroplasms, we analyzed their formation in siRNA-treated, virus-infected cells. Viroplasms were visualized by immunofluorescence with an anti-NSP2 antibody. The results demonstrated that knocking down NSP5 expression heavily impaired the emergence of viroplasms, as can be seen in the representative images shown in Fig. 2(a). The effect was highly strain specific: si/SA11 and si/OSU strongly inhibited the emergence of viroplasms in cells infected, respectively, with SA11 (75–80% inhibition) and OSU (80–83% inhibition), while cells infected with the non-homologous strains were fully competent in sustaining viroplasm assembly. Moreover, the few viroplasms remaining in siRNA-treated cells appeared smaller and reduced in number. This was due to the lack of NSP5, and not NSP6, since OSU-NSP5 was able to complement viroplasms formation in si/SA11-treated cells infected with SA11 virus. This was shown using C7 cells, an MA104 stable transfectant expressing NSP5-OSU which is sensitive to si/OSU but not to si/SA11 (data not shown). These cells showed a significant increase, compared with MA104 cells, in the emergence of viroplasms when infected with SA11 following si/SA11 treatment (Fig. 2a). As expected, the same C7 cells did not rescue viroplasm formation when infected with OSU following si/OSU treatment (not shown). Using a fluoresceinated si/SA11 (si/SA11-fluo), we estimated that more than 80% of cells were transfected and unable to form viroplasms after SA11 infection, while infection with OSU was unaffected (Fig. 2b). The fluorescent si/SA11 was found as punctate foci in the cytoplasm adjacent to the nucleus, a localization characteristic of siRNAs (Kapadia et al., 2003).

The strong effect of siRNA treatment on NSP5 levels and viroplasm formation was correlated with a lack of cytopathic effect observed in cells infected for 15 h with SA11 and treated with si/SA11 but not with si/OSU (Fig. 3a), suggesting impaired virus replication. To investigate this aspect further, we analyzed production of infective virions and of viral dsRNA in SA11-infected or OSU-infected cells. Production of infective virions by siRNA-treated cells was visualized directly by infecting a stable transfectant cell line expressing NSP2–EGFP fusion protein, which shows, upon virus infection, relocation to viroplasms of the cytoplasmic diffused protein (Eichwald et al., 2004). Alternatively, the same virus inocula were used to infect MA104 cells and the appearance of viroplasms analysed by immunofluorescence with an anti-NSP2 serum.

As shown in Fig. 3(b), si/SA11, but not si/OSU, inhibited the production of infective SA11 virus. Analogous results were obtained with OSU infections in si/SA11- or si/OSU-treated cells (not shown). Virus titres were determined in cells infected with either virus strain and treated with the different siRNAs. As shown in Table 1, virus titres were reduced between 10- and 20-fold depending on the virus strain in cells treated with the corresponding homologous siRNA. It is worth noting that, since not all cells can be transfected with siRNA, a number of them will sustain virus replication. In different experiments, we observed that the reduction in OSU titres was always higher than that obtained with SA11, consistent with what we observed by immunofluorescence.

In agreement with these results, the synthesis of SA11 dsRNA, but not of OSU dsRNA, was greatly inhibited in cells treated with si/SA11. Similarly, si/OSU inhibited OSU but not SA11 replication (Fig. 4). Furthermore, as for the formation of viroplasms, complementation of SA11 dsRNA production was observed in si/SA11-treated C7 cells that express OSU-NSP5 but not for OSU replication following si/OSU treatment. This result also indicated that NSP6, as it is the case for OSU, is not essential for SA11 replication.

Taken together, the results presented revealed an essential role for NSP5 in the assembly of viroplasms and in virus replication.

**DISCUSSION**

The aim of our work was to determine the role of NSP5 in the context of virus replication. In virus-infected cells, NSP5 accumulates in viroplasms, while it has a diffused cytoplasmic localization when expressed in the absence of other viral proteins. NSP5, as well as NSP6, is encoded in gs11. However, in some viruses, NSP6 is completely absent (strains Alabama and Mc323) (Gorzgilia et al., 1989; Kojima et al., 1996) while, in the porcine OSU strain, a premature termination codon reduces the coding region to 51 residues (Gonzalez & Burrone, 1989). Since it is not yet possible to generate recombinant rotaviruses for a reverse genetic approach to study the function of viral proteins, we followed the RNA interference strategy. To knock down gs11 mRNA of two different rotavirus strains (simian SA11 and porcine OSU), a stretch of 19 nucleotides with a single nucleotide difference between the two strains was targeted with siRNAs specific for each of them. Both siRNAs were entirely specific, abolishing expression of NSP5 only for the virus encoding the fully homologous mRNA. This fact provided important internal controls for the siRNAs effects, ruling out a general alteration of siRNA-treated cells, since they were able to sustain replication of the viral strain with the non-homologous gs11 mRNA to a level comparable to that of control, untreated cells.

We found a strong inhibition of viroplasm formation in siRNA-treated, virus-infected cells. This result was in complete agreement with our recent finding on the inhibition of viroplasm assembly by specific anti-NSP5 intrabodies (Vascotto et al., 2004). Moreover, in line with the intrabody protein-knockout strategy, we also demonstrated that NSP5 and not NSP6 is the relevant protein for virus replication. Complementation was achieved with the NSP5 gene encoding a truncated version of NSP6 (OSU-NSP5). The phenotype of virus infection in NSP5-depleted cells resembled that of an NSP2 ts mutant, which was viroplasm- and
Fig. 2. Viroplasms in siRNA-treated cells. (a) Immunofluorescence of MA104 or C7 cells mock-transfected or transfected with si/SA11 or si/OSU and non-infected (Not inf.) or infected with the indicated virus strain. (b) MA104 cells transfected with fluoresceinated si/SA11 (si/SA11-fluo; green) and infected with the indicated virus strain. Viroplasms were visualized with an anti-NSP2 antibody (red). Nuclei were stained with Hoechst dye (blue).
replication-negative at the non-permissive temperature (Ramig & Petrie, 1984; Chen et al., 1990), as well as the phenotype of cells treated with RNA interference for NSP2 (Silvestri et al., 2004). Furthermore, the reduction of virus titres that we obtained was similar to what was found by RNA interference of NSP2 (Silvestri et al., 2004). These findings suggest that both NSP5 and NSP2 play structurally relevant roles in the assembly of viroplasms.

Table 1. Virus titres (f.f.u. ml⁻¹)

Virus titres were determined by immunofluorescence of MA104-infected cells with anti-NSP2 antibody.

<table>
<thead>
<tr>
<th>Infecting strain</th>
<th>siRNA</th>
<th>Virus titre (f.f.u. ml⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Expt 1</td>
</tr>
<tr>
<td>SA11</td>
<td>Mock</td>
<td>4.5 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>si/SA11</td>
<td>3.5 × 10⁵</td>
</tr>
<tr>
<td></td>
<td>si/OSU</td>
<td>3.6 × 10⁶</td>
</tr>
<tr>
<td>OSU</td>
<td>Mock</td>
<td>7.0 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>si/SA11</td>
<td>5.5 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>si/OSU</td>
<td>2.7 × 10⁵</td>
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Fig. 3. Production of infectious virions in siRNA-treated cells. (a) Cytopathic effect on si/SA11-treated (bottom left) or si/OSU-treated (bottom right) cells at 15 h post-infection with virus strain SA11 compared with mock-transfected uninfected cells (upper left) and to mock-transfected SA11-infected cells (upper right). (b) SA11 infective particles produced by untreated, si/SA11-treated or si/OSU-treated cells and detected by their ability to produce viroplasms upon infection of MA104 (upper row, anti-NSP2 red) or MA104 cells stably expressing NSP2–EGFP fusion protein (lower row, green). Nuclei were stained with Hoechst dye (blue).

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that most of the viral proteins found in virus-infected cells are, rather than the product of the internalized infecting virions, the consequence of the translation of mRNAs produced by de novo-formed particles (secondary transcription) that have not yet completed the final maturation steps, as has been shown for reovirus (Zweerink & Joklik, 1970; Acs et al., 1971; Sakuma & Watanabe, 1971; Skup & Millward, 1980). This interpretation is consistent with the active transcription of viral genes occurring in double-layered particles derived from purified mature infective virions (Bican et al., 1982; Kohli et al., 1993; Patton & Chen, 1999; Lawton et al., 2000; Thouvenin et al., 2001) and with the results of RNA interference for NSP2 (Silvestri et al., 2004). However, it has recently been suggested that subvirus replication intermediates, rather than double-layered particles, are responsible for secondary transcription (Lopez et al., 2005).

In conclusion, the data presented demonstrated that NSP5 is an essential protein for the formation of viroplasms and for virus replication and suggested that this non-structural protein can be considered a good target for therapeutic purposes. However, it remains to be determined whether NSP5, in addition to its crucial role in shaping the architecture of viroplasm, also participates directly in other steps of virus morphogenesis. The RNA interference approach, in combination with complementation assays with defined mutants, opens new opportunities to understand the function of viral proteins in the absence of a reverse genetic system.

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REFERENCES


