Rotavirus NSP5 phosphorylation is up-regulated by interaction with NSP2

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We have previously shown that a number of isoforms of the non-structural rotavirus protein NSP5 are found in virus-infected cells. These isoforms differ in their level of phosphorylation which, at least in part, appears to occur through autophosphorylation. NSP5 co-localizes with another non-structural protein, NSP2, in the viroplasms of infected cells where virus replication takes place. We now show that NSP5 can be chemically cross-linked in living cells with the viral polymerase VP1 and NSP2. Interaction of NSP5 with NSP2 was also demonstrated by co-immunoprecipitation of NSP2 and NSP5 from extracts of UV-treated rotavirus-infected cells. In addition, in transient transfection assays, NSP5 phosphorylation in vivo was enhanced by co-expression of NSP2. An NSP5 C-terminal domain deletion mutant, was completely unable to be phosphorylated either in the presence or absence of NSP2. However, a 33 aa N-terminal deletion mutant of NSP5 was shown to become hyperphosphorylated in vivo and to be insensitive to NSP2 activation, suggesting a regulatory role for this domain in NSP5 phosphorylation and making it a candidate for the interaction with NSP2. These mutants also allow a preliminary mapping of NSP5 autophosphorylation activity.

Introduction

Rotaviruses are important pathogens that belong to the family Reoviridae, causing diarrhoea in several species. Infective particles replicate in the cytoplasm of infected cells. Although several reports have described the characterization of rotavirus replication intermediates (Gallegos & Patton, 1989; Patton & Gallegos, 1989), molecular details of the replication mechanism remain unclear. Partially purified intermediate complexes (or sub-viral particles) obtained from SA11-infected cells were able to catalyse run-off synthesis of endogenous dsRNAs (Patton, 1986), as well as complete replication of exogenously added viral ss (+)-RNAs (Chen et al., 1994; Wentz et al., 1996; Patton et al., 1996; E. Fabbretti & O. R. Burrone, unpublished). These results showed that the viral RNA polymerase structural proteins VP1, VP2 and VP3 are essential components of the replicase particles (Patton et al., 1997; Prasad et al., 1996). VP2 was demonstrated to be an essential element for the replicase-transcriptase activity that appears to be involved in the organization of the viral genome (Lawton et al., 1997). In virus-infected cells replication appears to take place within the viroplasms, where two other non-structural rotavirus proteins, NSP2 and NSP5, are localized (Petrie et al., 1982, 1984; Welch et al., 1989). These discrete structures are found in the cytoplasm at 2–3 h post-infection (p.i.).

The NSP2 protein of 317 aa is encoded in gene segment 8 (in strain SA11) and possesses dsRNA and ssRNA binding capacity (Patton et al., 1993; Kattoura et al., 1992). Strains expressing temperature-sensitive mutants of this protein lose the ability to synthesize dsRNA and show a significant reduction in the synthesis of ssRNA (Chen et al., 1990). It has been shown that NSP2 is able to assemble in multimers and interact with the viral polymerase, VP1 (Kattoura et al., 1994). NSP5 is encoded in the longer ORF of genomic segment 11. It is a glycosylated phosphoprotein of 198 aa (in SA11) (Welch et al., 1989) with a high degree of conservation among different strains of group A rotaviruses including viruses with re-arrangements of genome segment 11 (Gianbiagi et al., 1994). The NSP5 primary sequence is characterized by an overall high content (24%) of serine and threonine. This is even higher (30.5%) in the 131-aa-long N-terminal portion which is followed by a highly charged basic-acid-basic (B-A-B) domain (60% charged residues) of 49 aa located between residues 131 and 179. The last 18 C-terminal aa are highly conserved even in group C viruses (Mattion et al., 1991). We have recently shown that NSP5 is hyperphosphorylated in vivo through a complex process, involving autophosphorylation, which gives...
rise to a number of different isoforms (26, 28 and 32–34 kDa) (Afrikanova et al., 1996). Similar results have also been obtained by other authors (Blackhall et al., 1997; Poncet et al., 1997). NSP5 is also characterized by having O-glycosidic mono-
saccharide residues of N-acetylgalactosamine (GalNAc) (González & Burrone, 1991), a modification that involves serine residues in several proteins of cytoplasmic and nuclear localization (for review see Haltiwanger et al., 1992).

Although NSP5 is found associated to cytoplasmic viro-
somes from the early stages of infection (2 h p.i.), no function has yet been assigned to it. In order to investigate NSP5 interactions with other proteins we performed in vivo protein cross-linking of virus-infected cells and analysed NSP5 immunoprecipitates. Here we show that NSP5 interacts with two other rotaviral proteins, NSP2 and VP1, and that NSP2 plays a key role in the hyperphosphorylation of NSP5.

Methods

- Cells, viruses and antibodies. MA104 cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal calf serum, 2 mM l-glutamine, 50 mg/ml gentamycin (Gibco). Rotavirus simian SA11 strain was propagated and grown in MA104 cells as described (Estes et al., 1979). Guinea pig anti-NSP5 serum was produced as described (González & Burrone, 1991). Mouse anti-NSP2 serum was obtained by the DNA-immunization technique (Raz et al., 1994). The complete NSP2 coding region was cloned in-frame downstream of a signal secretion leader peptide sequence in pUT-SEC plasmid (González et al., 1994). The complete NSP2 coding region was cloned in-frame downstream of Thr-130. NSP5 and mutated cDNA fragments were subcloned in pcDNA3 vector (Invitrogen) under the T7 promoter to generate pT7v-NSP5, pT7v-NSP2 and pT7v-∆NSP5. The NSP2 complete coding region was obtained by RT–PCR using total RNA preparation from SA11-infected cells and transferred to pcDNA3 (pT7v-NSP2). All amplified fragments of NSP5 mutants and NSP2 were cloned into pUC18 and sequenced with pUC forward and reverse primers before transferring to the pcDNA3 vector.

- Radiolabelling with [35S]methionine, immunoprecipitation and PAGE analysis. Labelling of cells with [35S]methionine was performed essentially as described by Afrikanova et al. (1996). Total cellular extracts were prepared in TNN buffer (100 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.5% NP40, 1 mM PMSF) and used for immunoprecipitation and PAGE analysis as described (Afrikanova et al., 1996).

- Chemical DSP cross-linking. Cell monolayers were washed twice with PBS containing 1 mM Mg2+ and 1 mM Ca2+, overlaid with 1.5 ml washing buffer and dithiobis(succinimidyl)propionate) (DSP) chemical cross-linker (Pierce) was added to the cells at a final concentration of 200 µM. The cells were then incubated at 4 °C for 15 min when the medium was removed. After washing with PBS the cross-linker was quenched twice with 1.5 ml 50 mM Tris pH 7.5, 150 nM NaCl buffer for 5 min at 4 °C. Cellular extracts were prepared in 100 µl TNN.

- UV treatment of living cells. For UV treatment of cell monolayers, the medium was removed and the cells were washed twice with PBS, overlaid with 1 ml TBS buffer and exposed to UV-Stratalinker 1800 for 3 min (total dose 486 µJ) on ice. The distance between the cell monolayer and the UV lamp was kept at 15 cm. The extracts were prepared in RIPA buffer. Extracts were spun at 10,000 g for 7 min and supernatants used for immunoprecipitation analysis.

Results

- In vivo chemical cross-linking. In order to investigate possible interactions of NSP5 with other cellular or viral components, we performed in vivo chemical cross-linking of virus-infected cells, followed by immunoprecipitation with an anti-NSP5 specific antibody. We used DSP as a chemical cross-linker since it permeates into living cells, is able to cross-link proteins interacting at distances of up to 12 Å, and is sensitive to reducing agents. Analysis of the immunoprecipitates derived from cross-linked virus-infected cell extracts showed two new proteins in addition to...
the different phosphorylated isoforms of NSP5 (Afrikanova et al., 1996). These two proteins migrated on SDS–PAGE identically to the viral proteins VP1 and NSP2 of molecular masses 120 and 35 kDa, respectively (Fig. 1a, lane 4; 1b, lanes 2–3). Other bands migrating like the two abundant viral proteins VP2 and VP6 were occasionally present in smaller amounts in the immunoprecipitates, but their presence was cross-linking-independent and varied according to the extent of washing of the immunoprecipitates.

When then analysed by non-reducing SDS–PAGE, NSP5 immunoprecipitates consist of a complex of high molecular mass, with most of the material remaining in the top of the gel, and some free NSP2 and NSP5 (Fig. 1b, lane 1). After reduction of the same immunoprecipitates, the three components become apparent: VP1, NSP2 and NSP5. A small proportion of both NSP2 and NSP5 was recovered from the immunoprecipitates even before treating with reducing agents (Fig. 1b, lane 1). While this indicates that part of NSP5 is not involved in interactions with other proteins, for NSP2 it suggests that this protein is less efficiently cross-linked to other protein(s) of the complex. Treatment of the complex with λ-phosphatase (λ-Ppase) before reduction and SDS–PAGE analysis produces the 26 kDa band of dephosphorylated NSP5 (Afrikanova et al., 1996), while the two other components of 35 and 120 kDa remain unchanged (Fig. 1c). This result confirms that all the bands smaller than 35 kDa corresponded to the different phosphorylated isoforms of NSP5.

Sequential immunoprecipitation with anti-NSP2 and anti-NSP5 antibodies of cross-linked extracts indicated that most of the NSP2 interacting with NSP5 seems to remain buried within the cross-linked complex not accessible to the precipitating antibody (data not shown).

**In vivo UV-treatment induces co-precipitation of NSP2 and NSP5**

Exposure of virus-infected live cells to UV light was also used as an alternative approach to detect NSP5 interactions. The UV-cross-linking technique is mainly used to detect protein–nucleic acids interactions in vivo (Budowsky & Abdurashidova, 1989). However, as seen in Fig. 2, UV treatment of rotavirus-infected cells induced co-immunoprecipitation of NSP2 and NSP5, regardless of whether the cell extracts were prepared immediately after the [35S]methionine-labeling pulse or following the 2 h chase. As expected a higher proportion of the 28 kDa NSP5 isoform was obtained after the chase period (Fig. 2a). In the experiment shown in Fig. 2(b), done with extract of cells labelled for 30 min, the isoforms of molecular masses higher than 28 kDa appeared as two sharper bands of 34 and 30 kDa in the immunoprecipitates from cross-linked cells (lane 1) when compared to those from non-cross-linked cells (lane 3). These two bands probably represent more homogeneously phosphorylated forms, since λ-Ppase treatment converted them all to the 26 kDa band, without affecting the migration of NSP2. Interestingly, while NSP2 clearly co-precipitated with anti-NSP5, no VP1 did so under these conditions. This result is particularly intriguing since NSP2 has been shown (by in vivo UV-cross-linking) to have ss- and dsRNA binding capacity. The co-precipitation of NSP2 could be the consequence of conformational modifications induced...
hyperphosphorylated-form bands was obtained, compared with the non-cross-linked cells (Fig. 3a, b, lanes 1 and 3). Since we and others have shown that NSP5 is itself a kinase with autophosphorylation activity (Afrikanova et al., 1996; Blackhall et al., 1997; Poncet et al., 1997), the results shown suggest that the association of NSP5 with NSP2, VP1 or both has an activating effect on the kinase activity of NSP5 or on NSP5 as the substrate of phosphorylation. While the immunoprecipitates obtained from the cross-linked extracts represented a complex (or complexes) of three proteins, VP1, NSP2 and NSP5, neither VP1 nor NSP2 appeared to be phosphorylated in the in vitro assay. As shown in Fig. 3(b), lanes 2 and 4, λ-Pase treatment of the phosphorylated samples removed almost all the phosphate. The total amount of NSP5 in the immunoprecipitates subjected to the in vitro phosphorylation assay was the same in cross-linked and non-cross-linked extracts, as evidenced by the λ-Pase treatment (Fig. 3a, lanes 2 and 4). It is noteworthy to mention that we have also confirmed that neither VP1 nor NSP2 is phosphorylated in vivo (data not shown).

**NSP2 activates phosphorylation of NSP5 in vivo**

The possibility of NSP2 alone being sufficient for the activation of NSP5 hyperphosphorylation was directly tested in vivo, in experiments in which NSP5 and NSP2 were co-expressed in the absence of any other rotaviral protein. We used the T7–vaccinia virus expression system, co-transfecting plasmids containing the complete coding region of NSP5 and NSP2 (pT7v-NSP5 and pT7v-NSP2) under the control of the T7 polymerase promoter into MA104 cells. Total extracts of transfected cells previously infected with T7–vaccinia were
analysed by Western immunoblotting to visualize the different NSP5 isoforms. Fig. 4 shows the results of two independent experiments. Expression of NSP5 alone gives rise to the 26 kDa and, to a lesser extent, 28 kDa bands and no other NSP5 product. This is in complete agreement with our previous observations on NSP5 phosphorylation in MA104 stable transfectants (Afrikanova et al., 1996). On the other hand, when both NSP2 and NSP5 were co-expressed, a clearly increased phosphorylation (28 kDa band) and hyper-phosphorylation (32–34 kDa bands) of NSP5 were obtained. As expected all these bands were sensitive to λ-Ppase. This result demonstrated that NSP5 hyperphosphorylation is activated in vivo by NSP2, and suggested a direct interaction of NSP2 and NSP5 in the absence of any other viral protein. It remains to be determined whether the up-regulation of phosphorylation is the consequence of an increased kinase activity of NSP5 or NSP5 becoming a better substrate.

A regulatory role of the NSP5 N-terminal domain on phosphorylation

Two deletion mutants of NSP5 were constructed, lacking, respectively, the 33 aa of the N-terminal portion (ΔN33) or the 68 aa of the C-terminal domain (ΔC68) (Fig. 5a). In the case of ΔN33, Ile-34 was changed to Met. The resulting protein contains 165 aa with an apparent molecular mass of 22 kDa upon reducing PAGE of the in vitro translated product. The ΔC68 mutant of 130 aa (apparent molecular mass 18 kDa), lacks the B-A-B domain (aa 130–179) and the highly conserved terminal 20 aa.

When ΔN33 was expressed in vivo by transfection using the T7–vaccinia system, a strong phosphorylation was observed with the appearance of several bands of apparent molecular mass higher than 22 kDa (filled arrowheads in Fig. 5). All these bands corresponded to hyperphosphorylated forms, as revealed by their sensitivity to λ-Ppase (Fig. 5d). These data suggest that the lack of the 33 aa N-terminal domain allows NSP5 to become hyperphosphorylated. Interestingly, co-expression of NSP2 and ΔN33 did not produce any further activation of phosphorylation (Fig. 5c). On the other hand, the ΔC68 mutant was not phosphorylated at all, even when co-expressed with NSP2. Taken together, these results indicate that the N-terminal 33 aa domain plays a regulatory role in the phosphorylation of NSP5, by reducing the ability of the complete molecule to become hyperphosphorylated when expressed in vivo in the absence of other viral proteins. This domain probably also regulates the proportion of higher molecular mass isoforms in infected cells.

Discussion

We have previously shown that the rotavirus non-structural protein NSP5 is hyperphosphorylated during the virus replicative-cycle. As a consequence, a heterogeneous set of hyperphosphorylated forms with molecular masses of up to 32–34 kDa are produced in addition to the main isoforms of 26 and 28 kDa. NSP5 appears to have autophosphorylation...
activity in the absence of any other viral or cellular protein (Afrikanova et al., 1996; Blackhall et al., 1997; Poncet et al., 1997). The 26 kDa protein, expressed in E. coli and purified, was autophosphorylated in an in vitro kinase assay (Blackhall et al., 1997; Poncet et al., 1997). However, we have shown that NSP5 obtained by in vitro translation, becomes phosphorylated and transformed into the 28 kDa form only when extracts from infected cells were added to the assay. Moreover, hyperphosphorylation of the 32–34 kDa forms in vitro can be obtained using NSP5 protein derived from virus-infected cells (Afrikanova et al., 1996). These results supported the idea that phosphorylation of NSP5 is a complex process that initiates with the transformation of the 26 kDa precursor into the 28 kDa form which, in virus-infected cells, is hyperphosphorylated giving rise to the 32–34 kDa isoforms. This suggests that control of NSP5 phosphorylation may be regulated through interactions with other proteins.

We have now demonstrated that NSP5 hyperphosphorylation can also take place in vivo when NSP5 is co-expressed with NSP2. In addition, we demonstrated, by cross-linking and immunoprecipitation, a direct interaction of NSP5 with NSP2 and the viral polymerase, VP1.

NSP2 is a rotaviral non-structural protein which has been previously found to interact also with the viral polymerase and to form homomultimers (Kattoura et al., 1994). NSP2 binds to viral ssRNAs and can be cross-linked in vivo to the 11 dsRNA genomic segments. The 10S homomultimers possess ssRNA binding activity as well. Therefore, it is possible that the interaction(s) between NSP5 and NSP2 is facilitated when the latter is bound to RNA, as suggested by the UV-treatment experiments. It has been demonstrated that under the same conditions used in our experiments, UV induces cross-linking of NSP2 to RNA (Kattoura et al., 1992). It is therefore possible that following UV treatment, NSP2 may acquire a conformation that favours and stabilizes a non-covalent interaction with NSP5. This association does not involve disulphide bonds since the two proteins separated in PAGE under non-reducing conditions (not shown).

An interesting observation is that the viral polymerase, VP1, co-precipitates with NSP5 from DSP-cross-linked extracts, suggesting that it could also be an additional component of NSP2–NSP5 complexes. However, we obtained different results regarding the presence of VP1 in immunoprecipitates from DSP-cross-linked or UV-treated extracts. The fact that VP1 does not co-precipitate from UV-treated extracts suggests that the nature of the NSP5–NSP2 and NSP5–VP1 interactions is different.

The NSP2–NSP5 interaction appears to have functional consequences regarding the level of phosphorylation of NSP5. A first indication of this fact was obtained in the in vitro phosphorylation assays of DSP-cross-linked extracts, which was later confirmed by co-expression of both proteins in vivo. In the latter case, the NSP5-phosphorylation pattern obtained was very similar to that observed in infected cells. These results demonstrated that NSP2 up-regulates the phosphorylation of NSP5 in vivo.

Even though we cannot rule out a role for cellular kinases in NSP5 phosphorylation, we favour the idea that a direct interaction between NSP2 and NSP5 is essential for NSP5 hyperphosphorylation.

Many kinases become activated by phosphorylation by other kinases (Superti-Furga & Courtneidge, 1995; Cooper & Howell, 1993). In the present case, NSP5 hyperphosphorylation could be the consequence of an indirect effect of NSP2 on cellular kinases. However, our results support the idea of a direct interaction because: (a) NSP2 co-immunoprecipitated with NSP5 following chemical cross-linking and UV treatment of infected cells; (b) NSP2 enhanced the hyperphosphorylation of NSP5 32–34 kDa bands in vitro phosphorylation experiments; and (c) co-expression of NSP2 and NSP5 induced the formation of the 32–34 kDa phosphorylated isoforms in cells that would have otherwise expressed only the 26 and 28 kDa forms.

The results obtained with the AN33 and ΔC68 deletion mutants of NSP5 also support the hypothesis of an interaction with NSP2. While deletion of the N-terminal portion of NSP5 contributed to an enormous increase in phosphorylation as compared to the wild-type NSP5, deletion of the C-terminal domain completely abolished NSP5 phosphorylation, even in the presence of NSP2. This reinforces the hypothesis that NSP5 is autophosphorylated in vivo, since ΔC68 lacks the highly conserved 178–198 region, with homology to guanido kinases (Mattion et al., 1994), but contains the most serine- and threonine-rich portion of the molecule.

The inability of NSP2 to activate phosphorylation of ΔN33 suggested that this domain could be important for the NSP2–NSP5 interaction. We hypothesize that the N-terminal portion of NSP5 plays the role of an inhibitory domain of NSP5 hyperphosphorylation. Up-regulation of this activity could be induced by conformational changes resulting from the interaction of NSP5 with NSP2. Such interaction has been also reported using a yeast two-hybrid system (Poncet et al., 1997). In addition, the results with the ΔN33 and ΔC68 mutants suggest that the putative kinase activity maps to the C-terminal region of NSP5. Interestingly, although the 33 aa N-terminal domain has the highest content of Ser and Thr (48%), its absence does not preclude NSP5 from becoming hyperphosphorylated, indicating that the sites of such phosphorylation reside in other regions of the protein.

The three proteins that we found to interact (NSP2, NSP5 and VP1) are present in the viroplasms of infected cells. NSP2 has been reported to interact with VP1 (Kattoura et al., 1994). Recently, using a monoclonal anti-NSP2 antibody, a viral RNA–protein complex with replicase activity was recovered (Aponte et al., 1996). This complex contained the structural proteins VP1, VP2, VP6, and NSP2, and was able to complete nascent RNA negative-strands, but did not use exogenous RNAs as templates (Patton, 1996). Other authors have shown
that, in vitro, replicative complexes able to uptake exogenously added viral ss (+-)RNAs contain, among other viral proteins, NSP2 and NSP5 (Patton et al., 1996; E. Fabbretti & O. R. Burrone, unpublished). Numerous phosphoproteins have been implicated in the replication of other RNA viruses as well (Kapoor et al., 1995; Kann et al., 1995; Chang et al., 1994; Lastarza et al., 1994). Our finding that NSP5 consists of a wide range of phosphorylated isoforms, with phosphorylation being up-regulated by NSP2, suggests a possible involvement of NSP5 in the process of sorting and packaging of the viral RNA during the replicative cycle.

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References


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