Phosphorylation generates different forms of rotavirus NSP5

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NSP5 (non-structural protein 5) is one of two proteins encoded by genome segment 11 of group A rotaviruses. In virus-infected cells NSP5 accumulates in the virosomes and is found as two polypeptides with molecular masses of 26 and 28 kDa (26K and 28K proteins). NSP5 has been previously shown to be post-translationally modified by the addition of O-linked monosaccharide residues of N-acetylglucosamine and also by phosphorylation. We have now found that, as a consequence of phosphorylation, a complex modification process gives rise to previously unidentified forms of NSP5, with molecular masses of up to 34 kDa. Treatment with phosphatases of NSP5 obtained from virus-infected cells produced a single band of 26 kDa. NSP5 could be phosphorylated in vitro by incubation of immuno-precipitates with [γ-32P]ATP, producing mainly phosphorylated products of 28 and 32–34 kDa (32–34K). In both in vivo and in vitro phosphorylated NSP5, phosphates were only found attached via serine and threonine residues. The in vitro translated NSP5 precursor polypeptide, molecular mass 25 kDa (25K), could also be phosphorylated and transformed into a 28K protein by incubation with extracts obtained from virus-infected cells, but not from non-infected cells. In addition, NSP5 labelled in vivo with [1,6-3H]glucosamine showed mainly the presence of the 26K and 28K proteins (converted to 26K by protein phosphatase treatment) suggesting that the type of protein produced is regulated according to the level of phosphorylation and/or O-glycosylation. The results also suggest that NSP5 is autophosphorylated.

Introduction

Rotaviruses, members of the family Reoviridae, have a genome composed of 11 segments of double-stranded RNA that encode six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) (Estes et al., 1983; Mattion et al., 1994; McCraw & McCorquodale, 1982; Patton, 1995). Of all the rotavirus proteins, five have been shown to bear post-translational modifications. VP2 and VP6 appear to be myristylated (Clark & Desselberger, 1988), while both VP7 and NSP4 (NS28) contain N-linked high-mannose oligosaccharide residues, a finding consistent with their exclusive localization in the rough endoplasmic reticulum (Kabcenell & Atkinson, 1985). NSP5 (previously known as NS26), the product of the longer open reading frame of genome segment 11 (Mattion et al., 1991), is a protein of 198 aa (strain SA11), with a high content of serine (21%) and threonine (4-5%), which is localized in virosomes of virus-infected cells (Welch et al., 1989). NSP5 is highly conserved among different strains of group A rotaviruses and in viruses with rearrangements of genome segment 11 (Giambiagi et al., 1994; Gonzalez et al., 1989; Mattion et al., 1988). NSP5 was originally described as having an apparent molecular mass of 26 kDa in SDS–PAGE (Ericson et al., 1982; Mason et al., 1980); this was later demonstrated to be a precursor of a more mature form of 28 kDa (Gonzalez & Burrone, 1991; Welch et al., 1989). Two post-translational modifications of NSP5 have been described: phosphorylation (Welch et al., 1989) and glycosylation, the latter by addition of O-linked monosaccharide residues of N-acetylglucosamine (Gonzalez & Burrone, 1991), a modification present in proteins localized to the cytoplasmic and nucleoplasmic compartments of the cell (Hart & Haltiwanger, 1988; Hart et al., 1989). In the present study we describe an in vivo hyperphosphorylated form of NSP5 with an apparent molecular mass of up to 34 kDa according to the level of phosphorylation.

Methods

- Cells and viruses. MA104 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM-l-glutamine, 50 μg/ml gentamycin (Gibco). Rotavirus simian SA11 and porcine OSU strains were propagated and grown in MA104 cells as previously described (Estes et al., 1979). The C7-MA transfectant cell line was obtained by transfecting MA104 cells with pKG4-11 plasmid containing the complete coding region of the Osu rotavirus NSP5 (Gonzalez & Burrone, 1989) under the control of the...
SV40 promoter region. Transfectants were selected by resistance to 500 µg/ml G418 (Geneticin; Gibco).

**Western immunoblot analysis.** Lysates of infected and mock-infected MA104 cells (corresponding to 0.5 x 10⁵ cells) were resolved on 12% SDS-polyacrylamide gels (Laemmli, 1970). Proteins were transferred onto a nitrocellulose membrane and reacted to a guinea-pig anti-NSP5 serum (Gonzalez & Burrone, 1991) as described (Batista et al., 1995).

**Radiolabelling of rotavirus-infected cells with [35S]methionine, [32P] and [1,6-3H]glucosamine.** Confluent monolayers of MA104 cells in 60 mm diameter Petri dishes were infected at high multiplicity (20–30 p.f.u. of trypsin-activated SA11 rotavirus per cell). After 3–4 h post-infection cells were incubated with methionine-free RPMI medium containing 50 µCi/ml (1000 Ci/mmol) [35S]methionine (Amersham). For pulse-chase experiments, the cells were labelled for 15 min in methionine-deficient RPMI medium containing 50 µCi/ml [35S]methionine. The labelling medium was removed and replaced with RPMI medium supplemented with 0.25 mg/ml methionine and further incubated for 2 h. For [32P]-labelling, cells were fed with 2 ml phosphate-free minimal essential medium for 30 min at 2.5 h post-infection and labelled by adding 600 µCi [32P] (Amersham) for 2 h at 37 °C. Labelling with [1,6-3H]glucosamine (52 Ci/mmol; New England Nuclear) was performed essentially as described (Gonzalez & Burrone, 1991). After labelling, cells were washed and lysed in 100 µl TNN lysis buffer (100 mM-Tris-Cl, pH 8.0, 250 mM-NaCl, 0.5% NP40, 1 mM-β-mercaptoethanol). Autoradiography was enhanced by fluorography using Amplify (Amersham). Autoradiography was performed at −70 °C using X-ray film (Kodak X-OMAT AR).

**Immunoprecipitations and PAGE analysis.** Ten µl of 35S- and 32P-labelled cellular lysates were diluted up to 100 µl with TNN buffer and subjected to immunoprecipitation by incubation with 1 µl of anti-NSP5 antiserum at 4 °C for 2 h. A 40 µl aliquot of 50% Protein A-Sepharose CL-4B beads (Pharmacia) in TNN buffer was added and further incubated for 60 min at 4 °C. Beads were washed three times with RIPA buffer (50 mM-Tris-Cl, pH 8.0, 150 mM-NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS). Antigen-antibody complexes were released by boiling in 2 x Laemmli sample buffer and analysed by electrophoresis on 12% SDS–PAGE. Visualization of 35S- and 3H-labelled proteins was enhanced by fluorography using Amplify (Amersham). Autoradiography was performed at −70 °C using X-ray film (Kodak X-OMAT AR).

**Phosphatase treatments.** For calf intestinal alkaline phosphatase (CIP) (New England Biolabs) treatments, immunoprecipitates were resuspended in 20 µl of reaction buffer containing 10 mM-Tris-Cl, pH 8.0, 50 mM-NaCl, 10 mM-MgCl₂, 1 mM-DDT, 1 mM CIP (10000 U/ml) and incubated at 37 °C for 1–3 h. Lambda protein phosphatase (λ-Pase) (New England Biolabs) treatment was performed by resuspending the immunoprecipitates in 40 µl of reaction buffer containing 50 mM-Tris-Cl, pH 7.8, 5 mM-DDTT, 2 mM-MnCl₂ 100 µg/ml BSA and 1 µl λ-Pase (400000 U/ml) and incubating at 30 °C for 1 h.

**‘In vitro’ phosphorylation.** Sepharose beads containing immunoprecipitates were washed twice with RIPA buffer and once with kinase buffer (50 mM-Tris-Cl, pH 8.0, 1.5 mM-mercaptoethanol, 5 mM-MgCl₂, 1 mM-DDT, 5% glycerol). The reaction was carried out in 20 µl kinase buffer containing 10 µCi (3000 Ci/mmol) [γ-32P]ATP (Amersham) for 30 min at 37 °C. Unincorporated [γ-32P]ATP was removed by two washes with RIPA buffer before protein elution.

**Phosphoamino acids analysis.** Phosphoamino acids were analysed by the method of Cooper et al. (1983). In vivo and in vitro 32P-labelled NSP5 was immunoprecipitated and resuspended in 5% m-HCl (Merck). Protein A–Sepharose beads were pelleted by centrifugation and the supernatant was hydrolysed for 2 h at 110 °C. Hydrolysates were dried under vacuum and dissolved in pH 1.9 buffer (99% formic acid–glacial acetic acid–H₂O, 44:4:156:1800) containing phosphoserine, phosphothreonine and phosphotyrosine (1 µg of each) as standards. Samples were applied to 10 x 20 cm cellulose thin-layer plates (Merck) and electrophoresed in pH 1.9 buffer at 1200 V for 25 min. After drying, a second dimension of electrophoresis in pH 3.5 buffer (pyridine–glacial acetic acid–H₂O, 10:100:1850) was performed at 2000 V for 40 min. Plates were dried, sprayed with ninhydrin and exposed to X-ray film for autoradiography.

**‘In vitro’ translation.** The protein-coding region of the NSP5 cDNA was subcloned downstream of the T3 RNA polymerase promoter in the transcription plasmid pSP6/T3 (BRL) to yield plasmid pT3-NSP5. The construct was transcribed using T3 RNA polymerase (Pharmacia) and the transcript translated in rabbit reticulocyte lysate (Promega). After 1 h of translation, extracts of non-infected or infected MA104 cells, or immunoprecipitated NSP5, were added and further incubated for 1 h at 37 °C. Translation products were immunoprecipitated with anti-NSP5 antiserum and subjected to λ-Pase treatment when indicated and analysed in 12% SDS–PAGE.

**Figures.** Autoradiograms shown in all figures were obtained by scanning with a StudioScan IIIsi (Agfa) scanner using Adobe Photoshop software run on a Power Macintosh 8100/100 computer.

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**Results**

**Multiple forms of post-translationally modified NSP5**

The rotavirus NSP5 protein produced in virus-infected cells was analysed by immunoblotting of electrophoresed total cellular extracts (Fig. 1a, lane 1) or by immunoprecipitation of the [35S]methionine-labelled material followed by SDS–PAGE (Fig. 1b, lanes 2, 3 and 4). As expected, two major bands of apparent molecular masses 26 and 28 kDa (26K and 28K) were obtained. However, additional material which appeared as a smear (and sometimes as a double band) with a molecular mass of up to 34 kDa ("34K") was also clearly detected in both cases (lanes 1 and 2). A similar pattern of bands was observed when NSP5 was immunoprecipitated from virus-infected cells labelled in vivo with [35S]methionine and [32P] (Fig. 1c, lanes 5 and 6). In this case, the 32–34K band became more evident while the 26K protein was labelled less. That these NSP5 forms were the consequence of phosphorylation was demonstrated by treatments of the immunoprecipitates with two different phosphatases, a recombinant bacterial serine/threonine and tyrosine λ protein phosphatase (λ-Pase) and alkaline calf intestine phosphatase (CIP). In both cases a main band of 26K was obtained (Fig. 1b, lanes 3, 4, 5 and 6). Although CIP treatment was also efficient in removing phosphates and reducing the size of the 32–34K protein, it was in general less effective, and produced a substantial amount of the 28K protein. The intensity of the 26K band increased when the protein was labelled with [35S]methionine, indicating a complete conversion to the small form. On the other hand, as expected, when NSP5 was labelled with [32P] most of the label associated with the 28K and 32–34K bands was lost, though a small amount was retained in the 26K form (Fig. 1c, lane 6). We have been unable to remove...
all the labelled phosphate from the 26K band even after prolonged and repeated treatments indicating that some of the phosphorylation sites are resistant to the phosphatases used.

These results indicated that part of the newly synthesized NSP5 in virus-infected cells was in a hyper-phosphorylated form. This conclusion was in agreement with the results obtained by immunoprecipitation of extracts of infected cells that had been labelled with a pulse of $^{35}$S-methionine for 15 min and then chased for 2 h. As shown in Fig. 1(d), a substantial amount of 32–34K form of NSP5 accumulated after the chase period (lane 8) while very little was detected immediately after the pulse (lane 7). In spite of this, the 28K form remained as the major product.

**In vitro** phosphorylation of NSP5

The results obtained in vivo prompted us to investigate the possibility of phosphorylating NSP5 in vitro. Fig. 2(a) shows that when immunoprecipitates of NSP5 were incubated solely with $\gamma$-$^{32}$P-ATP a characteristic pattern of phosphorylation was obtained that differed from the one obtained *in vivo* regarding the relative intensities of the various forms (lanes 1 and 2). The major product was represented by the 32–34K form, followed by the 28K form, while the 26K form was scarcely labelled (lane 1). No phosphorylation was obtained when the immunoprecipitates were derived either from infected cells using an anti-VP4 serum or from non-infected cells using an anti-NSP5 antibody (Fig. 2(b), lanes 4 and 5). NSP5 phosphorylation also took place with total cellular extracts from virus-infected cells instead of immunoprecipitates (data not shown). Treatment of *in vitro* phosphorylated NSP5 with $\lambda$-PPase also resulted in conversion into the 26K band conserving residual $^{32}$P (Fig. 2(b), lane 6). These results indicated that all the differentially phosphorylated NSP5 forms can still be further phosphorylated *in vitro.*
Fig. 4. Autoradiograms of the two-dimensional thin-layer electrophoresis corresponding to phosphoaminoacid analysis of in vivo (a) and in vitro (b) $^{32}$P-labelled NSP5.

Fig. 3 shows a similar in vitro phosphorylation with [γ-$^{32}$P]ATP, performed with extracts of cells that had been previously labelled with $^{35}$S methionine. The two panels correspond to the same gel exposed to detect $^{35}$S and $^{32}$P (a) and $^{32}$P only (b). The bands of 26, 28 and 32–34K from both panels are completely superimposable. CIP and $\lambda$-PPase treatments shifted the label from the 32–34K band towards those of the 28 and 26K forms (lanes 2) or completely into the 26K form (lanes 3), respectively.

Analysis of phosphorylated residues

The amino acid residues phosphorylated both in vivo and in vitro were determined by partial acid hydrolysis followed by two-dimensional thin-layer electrophoresis of the phosphoamino acids. As shown in Fig. 4, only phosphoserine and phosphothreonine were obtained in both cases. This result is not surprising considering the high content of serine and threonine (24.5%) of NSP5.

Since the change in electrophoretic mobility of NSP5 due to phosphorylation was very significant, we also analysed the soluble products obtained after partial alkaline hydrolysis (Cowling & Birnboim, 1994) of $^{32}$P-labelled NSP5. Only P$_i$ was obtained, thus ruling out the formation of polyphosphate chains as a consequence of the process of hyperphosphorylation (results not shown).

NSP5 phosphorylation in the absence of viral infection

We also investigated whether other viral proteins were involved in the phosphorylation of NSP5. For this purpose we derived a stable transfectant of the MA104 cell line (C7-MA) that constitutively expressed cytoplasmic NSP5. As shown in Fig. 5, the $^{35}$S methionine-labelled NSP5 immunoprecipitated from this cell line was composed of the two major 26K and 28K...
"in vitro" transl.: + - - + + 
\(\lambda\)-phosphatase: - - + - - + 

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Fig. 6. *In vitro* translation of NSP5. SDS-PAGE analysis of immunoprecipitates of NSP5 obtained by *in vitro* translation in a reticulocyte lysate system (lanes 1, 4, 5 and 6), and incubated with extracts of non-infected (lane 4) or virus-infected (lanes 5 and 6) cells. For comparison, immunoprecipitated *in vivo* [\(^{35}\)S]methionine-labelled NSP5 (lanes 2 and 3) was included. Samples treated with \(\lambda\)-PPase are indicated.

Fig. 7. Immunoprecipitation of NSP5 labelled *in vivo* with [1,6-\(^{3}\)H]acetylglucosamine for a period of 2 h (a) or 4 h (b). In (a), lanes 3 and 4 correspond to labelling with [\(^{35}\)S]methionine. Samples treated with \(\lambda\)-PPase are indicated.

Fig. 6

bands (Fig. 5, lane 1) while the material of higher relative molecular mass was hardly detected. Nevertheless, *in vivo* \(^{32}\)P-labelled NSP5 from C7-MA cells produced all isoforms (Fig. 5, lane 3), although their relative proportions were different from those observed in virus-infected cells. Treatment with \(\lambda\)-PPase produced, as expected, the 26K band (lanes 2 and 4). This result strongly suggests that no other viral protein is required for the post-translational phosphorylation of NSP5.

**Post-translational modification of ‘in vitro’ translated NSP5**

The product of *in vitro* translated NSP5 mRNA in a reticulocyte lysate system consisted of a precursor molecule with an apparent molecular mass of 25 kDa which was clearly different from the 26K band obtained *in vivo*, even after \(\lambda\)-PPase treatment (Fig. 6, lanes 1, 2 and 3). This mobility difference is most likely due to O-linked N-acetylglucosamine residues present in the 26K form (see below). When incubated with an extract of MA104 virus-infected cells, the 25K NSP5 precursor could be converted into a 28 KDa protein, while no effect was observed with extracts of non-infected cells (Fig. 6, lanes 4 and 5). The same conversion was also possible by addition of NSP5 immunoprecipitates, instead of total lysates, although with a lower efficiency (not shown). The change from 25K to 28K was demonstrated to be due to phosphorylation by its sensitivity to \(\lambda\)-PPase treatment (Fig. 6, lane 6).

**NSP5 O-linked glycosylation and phosphorylation**

We have previously shown that NSP5 is post-translationally modified by the addition of single residues of O-linked N-acetylglucosamine (Gonzalez & Burrone, 1991). \(^{[3]H}\)Glucosamine incorporation in virus-infected cells yielded the 26K and 28K NSP5 isoforms. The data presented here demonstrated that the changes in electrophoretic mobility (from 26 kDa to 28 kDa and 32-34 kDa) were due to the addition of phosphates which can be removed by phosphatases. To investigate O-glycosylation in the various NSP5 isoforms, virus-infected MA104 cells were metabolically labelled with [1,6-\(^{3}\)H]glucosamine, and the extracts immunoprecipitated with anti-NSP5 serum. Fig. 7(a) shows that, following a short period labelling (2 h), the [\(^{3}\)H]glucosamine label was distributed between the 26K and 28K bands with practically no label in the 32-34K forms (lane 2). However, longer labelling periods (4 h) with [\(^{3}\)H]glucosamine showed that some material of molecular mass greater than 28 kDa was produced (Fig. 7b, lane 5). Thus, the hyperphosphorylated forms (32-34K) appear to contain very little or none of the O-glycosidic residues. Nevertheless, a single band of 26K of increased intensity was obtained after \(\lambda\)-PPase treatment (lanes
1 and 6). These results demonstrated that the various in vivo forms of NSP5 have different relative contents of O-linked N-acetylglucosamine and phosphates and, in addition, that their differences in electrophoretic mobility were due to the degree of phosphorylation.

**Discussion**

We have shown that in virus-infected cells, as well as in the transfected cell line C7-MA, the rotavirus NSP5 undergoes a complex process of post-translational phosphorylation. In addition to the two previously identified 26K and 28K NSP5 forms, we have now found a new form of 32–34K, which is heterogeneous, judging from the smear observed in the autoradiographs. We also demonstrated that NSP5 can be phosphorylated in vitro, with incorporation of phosphates mainly in the 28K and 32–34K bands. Previous reports on NSP5 phosphorylation had indicated that the incorporation of phosphate did not modify the mobility of the protein in SDS–PAGE (Welch et al., 1989). We showed here that this is not the case, as two different phosphatases were able to convert NSP5 in the form of 26K.

We had previously demonstrated that NSP5 was also post-translationally modified by the addition of O-linked monomeric residues of N-acetylglucosamine and that β-elimination of [35S]methionine-labelled NSP5 produced a complete conversion of the 28K protein into a smaller 25K form (Gonzalez & Burrone, 1991). The removal of phosphate groups from NSP5 could have also contributed to this change of apparent molecular mass, as they are sensitive to alkaline hydrolysis (Juhl & Soderling, 1983; Offenbacher & Kline, 1984). We now confirmed (Fig. 7) that, as reported previously, the 26K band was labelled when cells were pulsed with [3H]glucosamine, thus indicating that this small form was modified by O-glycosylation. The results of λ-PPase treatment of [3H]glucosamine-labelled NSP5 demonstrated that all forms can be converted into the 26K form with no loss of carbohydrate moieties. However, the 32–34K form was labelled very little with [3H]glucosamine suggesting that the level of O-glycosylation is dependent on that of phosphorylation; this may indicate complementary roles for both types of post-translational modification (Haltiwanger et al., 1992). The fact that only a 26K band (and not 25K) is obtained after λ-phosphatase treatment of NSP5 produced in vitro may be due to O-linked N-acetylglucosamine residues present in underglycosylated 32–34K forms or to the residual phosphate groups that are phosphate resistant. In brief, our data are consistent with a model in which the addition of phosphates rather than O-glycosylation is the cause of the different mobilities of the 26, 28 and 32–34K forms. The fact that the 26K form can itself be labelled with phosphate indicates that phosphorylation of particular sites does not produce a change in NSP5 mobility as described for other proteins (Grasser & Konig, 1992; Theron et al., 1994). Cytoplasmic O-glycosylation appears to modify only slightly the PAGE mobility of NSP5, as revealed by the difference between the in vitro translated 25K precursor and the dephosphorylated 26K form immunoprecipitated from virus-infected cells. The 25K in vitro translated precursor could be converted into a 28K form when incubated with extracts of infected cells. The development of such an in vitro system will be useful to map the phosphorylation regions of NSP5.

The results presented suggest that, at least in part, NSP5 autophosphorylates. The evidence pointing in this direction is as follows: (a) there was phosphorylation in the immunoprecipitates of NSP5; (b) only cellular extracts from infected cells containing NSP5 (and not from MA104 non-infected cells), as well as NSP5 in the form of immunoprecipitate, were able to convert the in vitro translated 25K precursor into a 28K form; (c) NSP5 was post-translationally modified in the C7-MA cell line (ruling out the need for other viral proteins), while extracts of non-infected MA104 cells were unable to phosphorylate the 25K precursor. In addition, treating the immunoprecipitates with detergents, high salt and different pH was ineffective in separating the kinase activity from NSP5, and immunoprecipitates of NSP5 were able to phosphorylate exogenous added z-casein substrate (data not shown). Moreover, the region of NSP5 from Tyr-178 to the terminal Leu-198 (numbers corresponding to NSP5 SAll strain) has been indicated to be partially homologous to protein kinases (Matton et al., 1994). Based on the amino acid composition, two distinct regions can be identified in NSP5: the amino-terminal 130 aa domain with a high content (33%) of serine/threonine and 16% of the charged amino acids. Arg, Lys, Asp and Glu, and the carboxy-terminal domain of 67 aa with a very high content of charged amino acids (49%), no threonine and only 10% serine. We speculate that the 130 aa amino-terminal domain could be the region of hyperphosphorylation. Further work is needed to characterize this, as well as the putative NSP5 kinase domain.

The function of NSP5 in the rotavirus infective cycle remains unknown, nor is the role of the post-translational modifications well understood. The existence of phosphorylation and of O-glycosylation (both on serine residues) suggest a regulatory function for NSP5 (Haltiwanger et al., 1992). Since some of the NSP5 forms were of an unexpected size, we have investigated their presence in purified single-shelled and complete double-shelled virions, by Western immunoblotting. The negative results obtained (not shown) indicate that the NSP5 forms with greater molecular masses could also be considered as non-structural proteins.

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**References**


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